DEVELOPMENTAL MODIFICATIONS IN ARBACIA PUNCTULATA BY VARIOUS METABOLIC SUBSTANCES[1]

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Normal development is a sequela of normal cellular differentiation. In order to understand the precise programming in development, be it molecular or morphologic, attention must be given to the properties of the individual developing cell and its organelles and its metabolic events, as well as the overall susceptibility of response of the whole organism during development.

Major steps in the understanding of echinoid developmental biology have been made by experimental approaches which have brought about aberrations in development (see review by Lallier, 1964a) and more recently by biochemical analyses of these events (see review by Monroy and Maggio, 1964).

The present report concentrates on the response of the embryo as a whole organism to a variety of agents. By comparison with the normal sequences in development, these changes may provide insight into the mode of action, especially where a differential response is elicited.

The first experiments were based on Heath's (1954) observation that persistent nucleoli resulted when 1 to 1.5×10^{-5} gm./ml. cobalt chloride was added to cultures of chick heart and frontal bone. These persistent nucleoli remained in association with chromosomes during metaphase but were torn in two or adhered to one chromosome at anaphase. They remained in the cytoplasm despite reconstitution of new nucleoli in daughter cells. The early sea urchin experiments were designed to duplicate the unique cobalt effect, thereby, hopefully, pinpointing critical stages during early development (Mateyko, 1961). The prominent nucleolus, a characteristic feature of the large germinal vesicle in the oocyte of *Arbacia* has always attracted attention. To illustrate, recently, Esper (1965) discussed the cytochemistry of its vacuole. But the information on nucleoli in blastomeres of echinoids is difficult to assess. In fact, it was only recently that the karyotype of *Arbacia punctulata* was published (Auclair, 1965). German (1964) demonstrated that of the 44 chromosomes, two pairs were considerably larger. Whether these are the nucleolar-organizer chromosomes or even whether nucleoli are organized into compact bodies associated with specific chromosomes in the early cleavage stages is not established. Cowden and Lehman (1963) demonstrated that there were no nucleoli in the blastula of *Lytechinus verigatus* (*sic*) or *Melita quinquesperforata* (*sic*). It was only at the early gastrula, 12 hours post-fertilization, that nucleoli became visibly organized. Is "blastulation" used in the sense of the onset of asynchrony, and this inception of differentiation a critical moment in nucleolar phe-

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nomena? Periodically, the view is reiterated that rapidly dividing cells (as in early cleavage of *Fundulus; vide,* Richards and Porter, 1935) are anucleolate, and organized nucleoli which appear at the blastula or gastrula stage are evidence of heightened synthetic processes in interphasic nuclei. It was thought that the induction of persistent nucleoli might be informative and thus cobalt glycine and cobalt chloride were tried. It was equally important to consider the possibility of reversing this effect with RNA or RN-ase, and since RNA synthesis dependent on DNA led to the study of the effects of DNA and DN-ase, the chain reaction extended as an evaluation of over a dozen substances at different dose levels and even more combinations (Mateyko, 1965). To avoid repetition and an unwieldy manuscript, the literature on control mechanisms and the levels at which metabolites affect the activities of macromolecules by specific interaction at the level of DNA, RNA, and protein synthesis appears in the discussion of the results.

Materials and Methods

The echinoderms, *Arbacia punctulata,* were dredged from the marine waters about Woods Hole, Massachusetts, during the summers of 1961, 1962 and 1963. Experiments were also repeated with *A. punctulata* obtained from the New York Aquarium through the courtesy of the Director, Dr. Ross Nigrelli. The sexes were separated (sex determined by electrical stimulation) and maintained in the laboratory in running sea water at a temperature of about 20° C. Healthy urchins moved about actively and voraciously ate *Laminaria.*

Ovulation and shedding of sperm were induced by electrical stimulation (Harvey, 1954). Viable eggs were fertilized by suspensions of sperm in sea water. Zygotes were placed in Syracuse dishes containing sea water and kept at 20° C. Observations were made at close intervals as to normal progress of development. The elevation of the fertilization membrane, patterns of cleavage, and rate and modification of growth were studied. Since the experiments were designed to test the effect of various substances on development *per se,* usually zygotes were used as the initial starting point. The fertilized eggs (incipient two-cell stage) were transferred to the experimental solutions in covered stender dishes. These were kept immersed in running sea water at a temperature of 20° C. for the duration of the experiment.

The substances, alone and in combination, were in solution and added to the sea water environments. The substances were: ethanol and methanol, reagent grade; cobalt chloride, C. P.; RNA (reagent grade), RN-ase (crystalline), DNA (sperm, not polymerized) DN-ase (70,000 Dornase units/mg.), sodium taurocholate, and coumarin from Nutritional Biochemicals Corp., Cleveland, Ohio; thymine, cobalt glycine, C. P., mescaline sulfate, papain (purified), and trypsin (1:300) from Mann Research Lab., New York, N. Y.; pronase (B grade), Calbiochem, Los Angeles, Cal.; actinomycin D provided through the courtesy of Merck, Sharp, and Dohme, Rahway, N. J.; thalidomide through the courtesy of Dr. Martin Kuna, Bristol-Myers, New York, N. Y.; 4-nitroquinoline N-oxide through the courtesy of Dr. P. O'B. Montgomery, Southwestern Texas Medical School, Dallas, Texas.

Components were added to the sea water in what approximated physiological levels; that is, the literature was consulted to determine what these levels were, if any, and pilot experiments were made to assess the physiological levels. Lethal,

inhibitory, or ineffective concentrations of these agents were determined and appear as part of the observations. The effects of ethyl and methyl alcohol were also checked since these were used as solvents in some instances.

Each set of experiments involving a series of chemical agents (plus controls) utilized zygotes obtained from the same genetic background (one female, one male). Each set of experiments was repeated several times always using zygotes of uniform genetic background for the entire set of experiments to insure valid comparisons of development.

At close intervals the living embryos were examined, generally hourly, at a magnification of 100 × and compared with the controls as to stage of development.

In order to facilitate a true comparison of the degree of retardation, acceleration, or modification of development by environmental factors, a series of normal developmental stages were identified and given a code name. This stage series appears in the Results.

Photographs of the developing urchins under normal or modified environmental conditions were taken with a Zeiss Photomicroscope on Kodak Plus X 35 mm. film.

Results

Stage series

Since it is well-known (Harvey, 1956) that temperature alterations may accelerate or retard development of *Arbacia,* all embryos in any experiment must be maintained at the same temperature throughout the duration of an experimental series. In order to facilitate a valid comparison of the degrees and types of retardation, accleration, or modification of development brought about by various agents or environmental factors, a series of normal developmental stages at 20° C. has been identified by a code letter (Table I). Table I presents the usual sequential stages in the early development of *Arbacia punctulata.* In E. B. Harvey's (1956) book some of these have been charted, but the tabulation is not complete for any one temperature sequence.

The value of this series of stages is fourfold: (1) stages may be clearly identified by symbol; (2) precise stages are thereby stated (thus avoiding time factors which may lead to errors when environmental conditions such as temperature, crowding, etc., are present); (3) modifications in development brought about by experimental procedures are more readily interpreted when they can be compared with an unequivocal normal stage; and (4) more especially, brevity in citation.

When control and experimental embryos are raised at the same temperature within the normal range, and when the readings (that is, evaluation of developmental stages of both the controls and experimentals) are at the same time intervals, one needs only refer to the *stages* of development listed in Table I for a comparison of the progress of development. This is far preferable to expressing development in terms of *hours* of development, which often may be misleading because of the variability. No photographs of these stages are provided since Table I gives the description of each stage and photographs would be repetitions of the excellent and readily available Harvey (1956) illustrations.

All descriptions of development in subsequent tables are based on the stages listed in Table I, but amplification is made whenever abnormalities occur. In gen-

eral the following tables list only a brief spectrum of pertinent data to avoid the wealth of detail.

Alcohol

Since many water-insoluble substances (for example, actinomycin D) are dissolved in alcohols, before an aliquot was added to the medium, effects of ethanol

TABLE I

Designation of early developmental stages in Arbacia punctulata

Stage designation	Morphological appearance in normal development	Time from insemination	
		T = 23° C.*	T = 20° C.
IE	Immature ovarian and non-fertilizable egg (germinal vesicle)	−6 h.	−6 h.
E	Mature, haploid, unfertilized egg	0	0
FE	Fertilized egg (fertilization membrane, hyaline layer)	2 m.	5 m.
S	Streak (two pronuclei have fused and centrosomes form curved streak)	20–35 m.	35 m.
2C	Two defined cells (first cleavage)	50 m.	60 m.
4C	Four defined cells (second cleavage)	78 m.	100 m.
8C	Eight defined cells (third cleavage)	103 m.	140 m.
12C	Twelve defined cells (beginning fourth cleavage)	130 m.	170 m.
16C	Sixteen defined cells (completed fourth cleavage)	135 m.	178 m.
32C	Thirty-two defined cells (fifth cleavage)	167 m.	210–215 m.
M	Morula (sixty-four cells, sixth cleavage)	4 h.	4½–5 h.
EB	Early blastula (seventh and eighth cleavages, asynchrony)	6 h.	7–8 h.
B	Mid-blastula (*ca.* 500 cells)	—	9 h.
H	Hatched blastula (*ca.* 1000 cells, free of fertilization membrane)	7–8 h.	9½–11 h.
SB	Swimming blastula	—	12–15 h.
EG	Early gastrula (beginning invagination)	12–15 h.	15–16 h.
G	Gastrula (gut complete, skeleton forming)		17–19 h.
LGP	Late gastrula or prism (change of larval axis from ovoid to polygonal)	18–19 h.	21–23 h.
P1	Pluteus larva (pentagonal)	20 h.	23–25 h.
P2	Pluteus larva (bulge of arms appears)	22 h.	26–29 h.
P3	Pluteus larva (well-defined pluteus)	24 h.	30 h. +
P4	Pluteus larva (Long-armed, two-days old)	48 h.	55 h.
P5	Pluteus larva (maximum development, long-armed, before feeding)	72 h.	80 h. +
ML 1	Metamorphosing larva (+ one pair new arms)	11 d. +	12 d. +
ML 2	Metamorphosing larva (+ additional pair of arms)	21 d. +	24 d. +

* According to E. B. Harvey (1956), p. 97

D = dead organisms.

EX = exogastrulation.

and methanol were rechecked on developing embryos (Table II). Both ethanol and methanol at a concentration of 1% or less appeared to have no visible morphologic effect on development beyond a slight retardation which was slightly greater in ethanol. At 5%, development proceeded only to the morula stage. The displacement of pigment to a compact mass was quite noticeable in 5% ethanol. At concentrations of ethanol greater than 5%, alcohol acted as a fixative immobilizing the two-cell stage. Development in both 5% and 10% methanol proceeded

TABLE II

*The effects of ethanol and methanol on early development of A. punctulata**

Control Sea water	Experimental Sea water + alcohol							
All immersed as 2C	1% ETOH	5% ETOH	10% ETOH	25% ETOH	1% MEOH	5% MEOH	10% MEOH	25% MEOH
64C = M	M	M, pigment displaced	2C, D diffuse pigment	2C, D swollen	M	M	M, pigment displaced	2C, D fixed
LGP	LGP	M, D	—	—	LGP	M, D	M, D	—
P3	P2 sluggish	—	—	—	P2	—	—	—
P4	P3	—	—	—	P3	—	—	—

* Developmental stages at $t = 20°$ C. See Table I for descriptions.
D = dead embryos

only to the morula stage, with a clumping (dissolution) of echinochrome evident in 4 hours in 10% methanol. In the present experiments the levels of alcohol, where needed as a solvent (actinomycin D), were well below 1%.

Enzymes

One of the effects of trypsin, papain, pronase, and sodium taurocholate is on the cell surface. It is evident that trypsin attacks a cell-bonding substance, protein in nature, since disaggregation to blastomeres as well as dissolution of the fertilization membrane occurred in 4 hours. Development, however, did not proceed beyond the early blastula stage (Table III). In fact the blastomeres were digested.

TABLE III

*The effects of trypsin, papain, pronase, and sodium taurocholate on early development of A. punctulata**

Control Sea water	Experimental Sea water +						
All immersed as 2C	Trypsin 1 mg./ml.	Trypsin 2 mg./ml.	Papain 0.5 mg./ml.	Papain 1 mg./ml.	Pronase 2.5 mg./ml.	Na taurocholate 0.2 mg./ml.	Na taurocholate 2 mg./ml.
8C	8C	8C	8C	4C	8C[1]	8C	4C
M(64C)	M[1]	M[1]	M	8C	M	M	M
LGP	EB	EB, D	EB[2]	M, D	LGP	LGP	EB, D
P1	D				P1	P1	
P3			H, D		P3	P3	
P4					P4	P4	
P5					P5	P5	

* Developmental stages at $t = 20°$ C. See Table I for descriptions.
D = dead embryos.
[1] Fertilization membrane digested and disaggregation to blastomeres.
[2] Slight separation of blastomeres.

To maintain the blastomeres, the embryo must be removed to fresh sea water. They, however, did not reaggregate. Papain exerts a more drastic direct effect even at a concentration of 0.5 mg./ml., in that only a few zygotes become morulae, and still fewer reach the blastula. Separation to blastomeres occurred but was not especially striking. The fertilization membrane is more resistant here than in trypsin. Sperm remained motile in pronase (0.25 mg./ml.) even after two hours, but the motility was reduced by three hours. Eggs, both unfertilized and fertilized, exhibited a striking reduction in cortical consistency. The response of the fertilization membrane to pronase was not clearcut, for while 5% of the fertilized eggs had a distinct membrane, the remainder did not. It may, therefore, be inhibited or lysed. Lysis of a developed fertilization membrane occurred about 2 hours later. Development proceeded without the membrane. Separation of blastomeres occurred at 8C or 16C. The lack of a fertilization membrane obscured the decision

TABLE IV

*The effects of DNA on early development of A. punctulata**

Control / Sea water	Experimental / DNA			
All immersed as 2C	DNA 0.01 mg./ml.	DNA 0.02 mg./ml.	DNA 0.05 mg./ml.	DNA 0.1 mg./ml.
B				M
H	H		B	H
EG				EG
G	EG			
LGP	G, LGP	G, LGP	LGP	G, LGP
P1			P1	
P2	P1	P1	P2+	LGP, sluggish
P3	P2	P2	P2	LGP, D
P4	P3	P3	P3	
P5	P4	P4	P5	

* Developmental stages at t = 20° C. See Table I for descriptions.
D = dead embryos.

as to whether these cells can divide still further. Sodium taurocholate in a low concentration (0.2 mg./ml.) did not appear to damage the cells or retard development but at a concentration of 2 mg./ml. development ceased at early blastula, with swelling of the blastomeres and surface erosion. Survival in all instances was enhanced by washing at any time before lethal effects had set in.

DNA

At a concentration of 0.5 mg./ml. of DNA, development throughout is similar to that of the controls, for the embryos achieve the maximum development possible without the addition of food. At double the concentration, retardation in growth is striking and many specimens die. Skeletal formation continues in organisms which still maintain their original ovoid (gastrular) shape, often within a persistent fertilization membrane. The effect is reversible if specimens are removed and washed after 9 hours. Lower amounts of DNA, 0.01 to 0.02 mg./ml. do not alter appreciably the morphogenetic patterns or rate of development (Table IV).

DN-ase

Dn-ase added to sea water effects a consistent developmental pattern. At 4 μg./ml. the rate of development appears very slightly higher than in the controls (Table V). At 8 μg./ml. the effect is not detectable morphologically—the specimens look like the controls. In 0.05 mg./ml. the activity of the animals is curtailed —the movements are slower, while the rate of development is unaltered. Retardation of growth and activity is clear in 0.1 mg./ml. and more pronounced in 0.2 mg./ml., the animals dying in 48 hours. The morphology, however, is normal. The retarding effects can be reversed by removing the embryos from the medium even after 9 hours of development in 0.1 mg./ml. DN-ase.

TABLE V

*The effects of DN-ase on early development of A. punctulata**

Control / Sea water	Experimental / Sea water + DN-ase				
All immersed as FE	4γ/ml.	8γ/ml.	0.05 mg./ml.	0.1 mg./ml.	0.2 mg./ml.
Developmental stages					
B				B	
H		H			
SB	SB		SB, slow		
EG		EG		EG	
G				G	EB
LGP	LGP, P1	G, LGP	LGP	G, LGP	EG
P1	P2		P1		
P2		P1		P1	LGP
P3	P3+	P3	P3	P2	P1
P4	P4+	P4	P4	P3	P2
P5	P5+		P5	D	D

* Developmental stages at t = 20 °C. See Table I for descriptions.
D = dead embryos.

RNA

RNA was made up as a stock solution of 0.4 mg./ml. Stronger solutions (1 mg./ml.) cannot readily be made up in distilled water since the solubility is low and precipitation occurs. Solubilization by other means is likewise undesirable since it leads to degradation of the molecule.

The effects of RNA depend upon the dosage. At 0.1 mg./ml. and greater, retardation of growth occurs which is especially noticeable at gastrulation. Development through blastulation is, however, normal and no anomalies occur (Table VI). In 0.04 mg./ml. RNA, a slight acceleration of development up to gastrulation is evident, but it slows down subsequently to less than normal. Thus at lower concentrations of RNA the effect is one of enhancement of development. With a medium containing 16 to 20 μg./ml. the effect is imperceptible morphologically, for development proceeds at a normal pace and the embryos are in excellent condition at all stages. With 10 μg./ml., the early acceleration of development is most

TABLE VI

*The developmental effects of RNA on early development of A. punctulata**

Control Sea water	Experimental RNA						
All immersed as 2C	RNA 4 μg./ml.	RNA 8 μg./ml.	RNA 10 μg./ml.	RNA 16 μg./ml.	RNA 20 μg./ml.	RNA 0.04 mg./ml.	RNA 0.1 mg./ml.
B	B	B			B		B
H			EG			G	
SB	SB	SB	G		SB		SB
LGP	G	G, LGP		G		G, LGP	G
P1			P2		P1		LGP
P2			P2		P2	P1	P1
P3	P3	P2	P3	P2	P3	P2	P2
P4		P3	P4	P4	P4	P3	P2
P5	P5	P5					

* Developmental stages at $t = 20°$ C. See Table I for descriptions.

clear—the embryos gastrulate in advance of the control specimens. By 24 hours and subsequently, the spurt in development is less evident. Thus, it is questionable whether augmentation of sea water with 4 to 8 μg./ml. of RNA is effective in bringing embryos earlier to metamorphosis (Table VI). Washing the embryos free of the medium after development for several hours (5 to 9 hours) does not reduce the effect.

RN-ase

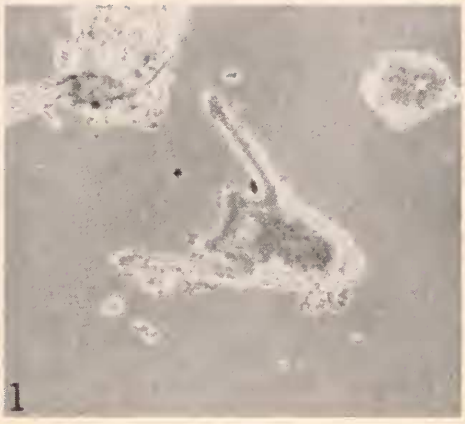
Unlike RNA, RN-ase is readily soluble. Stock solutions of 1 mg./ml. and 1.2 mg./ml. were prepared with distilled water.

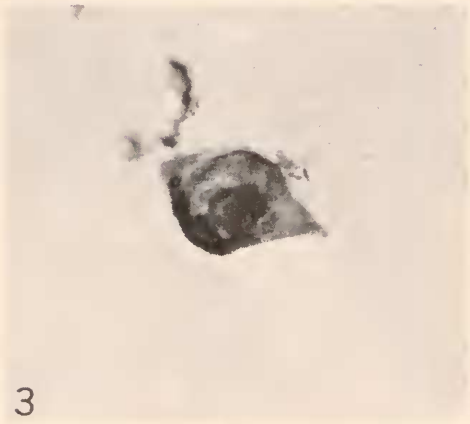
TABLE VII

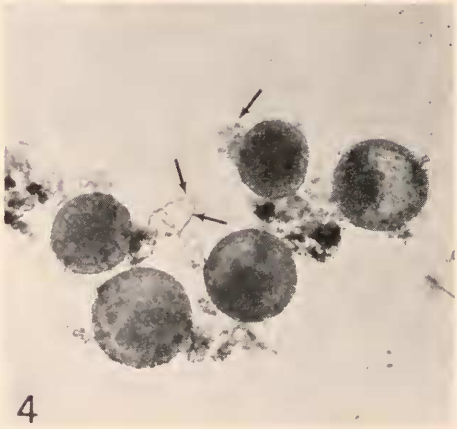
*The effects of RN-ase on early development of A. punctulata**

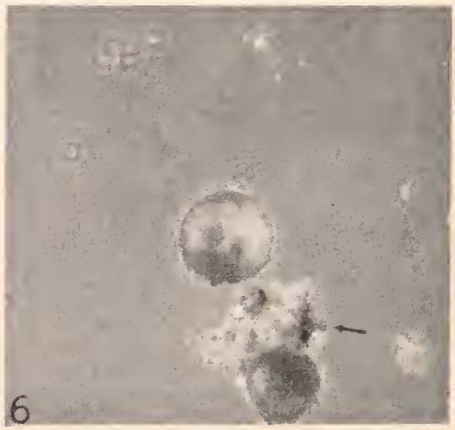
Control Sea water	Experimental Sea water + RN-ase					
All immersed as 2C	RN-ase 1.2 μg./ml.	RN-ase 12 μg./ml.	RN-ase 0.04 mg./ml.	RN-ase 0.05 mg./ml.	RN-ase 0.1 mg./ml.	RN-ase 0.12 mg./ml.
B	B				B	
H				H	H	H
SB	SB			SB	SB	SB
EG					EG	EG
G	G			G	G	
LGP	LGP	LGP	LGP	LGP	LGP	G, LGP
P1	P1			P1		G, LGP
P2						P1
P3	P3+	P3	P3	P3	P2	P2
P4	P4	P4	P4	P4	P3	P3
P5	P5		P5	P5	P4	P4

* Developmental stages at $t = 20°$ C. See Table I for descriptions.









FIGURES 1–6.

In RN-ase, the developmental effects follow a consistent pattern. With 1.2 μg./ml., to 0.05 mg./ml. an effect is lacking in that the embryos develop at a normal rate and appearance (Table VII). At 0.1 to 0.12 mg./ml. a slight retardation in the rate of growth is clear, the stages through gastrulation following a normal pattern. Embryos, however, are sluggish. Despite this, the inhibition is reversible if embryos are put into fresh sea water even after 9 hours development. No developmental anomalies were seen. Figure 1 illustrates a typical normal-appearing, long-armed pluteus (48 h.) raised in 0.1 mg./ml. RN-ase for 9 hours and then placed into fresh sea water.

Thymine

In low concentrations of thymine (0.04 mg./ml.) development proceeds normally but at a slower rate as if raised at a lower temperature. In 0.1 mg./ml. the retardation is exaggerated, and in 0.2 mg./ml. the retardation is even more drastic, and with 0.4 mg./ml. most of the embryos succumb at about blastulation (Table VIII). Malformations are rare. Gastrulation is noticeably interfered with at 0.4 mg./ml. since the embryos are moribund at this stage and are dead within 24 hours. The addition of RN-ase (0.5 mg./ml.) to thymine (0.1 mg./ml.) treated embryos does not significantly alter retardation brought about by thymine. With the addition of DN-ase (10 μg./ml.) to thymine (0.1 mg./ml.), the retardation which occurs is more evident after pluteus formation, since the well-formed plutei were small. Development through blastulation is normal in DNA (0.05 mg./ml.) plus thymine (0.1 mg./ml.) but an arrest occurs at the prism stage. Despite a persistent fertilization membrane, skeleton formation proceeds, but subsequently the embryos die. If embryos are washed free of the thymine, recovery is rapid.

Cobalt

An analysis of Table IX will show that in below lethal levels, the striking effect of cobalt chloride and cobalt glycine is the production of exogastrulae (Fig. 2). Where lethal effects were evident at stage 2C to 16C these were not tabulated. In general, the effect of both compounds is similar at a concentration of 0.02 mg./ml. If ova are fertilized in sea water containing cobalt salts, the effect arises sooner than if the 2C stage is used at the starting point.

FIGURE 1. *Arbacia punctulata* embryo at 48 hours; in RN-ase (0.1 mg./ml.) for 9 hours, washed; note excellent development of long-armed pluteus; phase, 145 ×.

FIGURE 2. Embryos reared in cobalt chloride (0.02 mg./ml.) for 3 days. Exogastrulation evident (arrow) and further differentiation inhibited; phase, 145 ×.

FIGURE 3. "Pollen grain" form induced by cobalt glycine (0.02 mg./ml.) after 3 days; 145 ×.

FIGURE 4. Note irregular alignment of cells (arrow) and pronounced exogastrulation (between arrows) in embryos raised in cobalt glycine (0.01 mg./ml.) for 48 hours. Change to pluteus form is inhibited; 145 ×.

FIGURE 5. Isolated spicules are reduced in size and deformed. 5A: cobalt glycine (0.02 mg./ml.) 48 hours. 5B: cobalt chloride (0.02 mg./ml.) 48 hours; phase, 180 ×.

FIGURE 6. Two-day-old embryo (cobalt chloride = 0.01 mg./ml.). Note large exogastrulate mass (arrow) with poor alignment of cells. Mass of cells emerges from spherical gastrula; phase, 145 ×.

Development in cobalt proceeds normally through the blastula although it is slowed down. In minimal levels (< 0.01 mg./ml.) embryos exhibit a growth retardation with sluggishly and erratically swimming forms. At higher concentrations (0.01 to 0.02 mg./ml.) the drastic exogastrulation produces such abnormal embryos ("pollen grain" forms) that normal plutei and metamorphosis cannot occur. Exogastrulation is preceded by a bloating which persists and results in a form that resembles a winged pollen grain (Fig. 3). In some (cobalt glycine, 0.01 mg./ml.) the surface is rough, owing to a lack of alignment of surface cells (Fig. 4). In all, however, spicule formation does occur. The skeleton is reduced to deformed microspicules in 0.01 mg./ml. cobalt glycine (Fig. 5a and b). Some malformations are so bizarre (cobalt glycine 0.02 mg./ml.) that a picture is pref-

TABLE VIII

*The effects of thymine on early development of A. punctulata**

Control Sea water	Experimental Sea water + Thymine				Thymine +		
All immersed as FE	0.04 mg./ml.	0.1 mg./ml.	0.2 mg./ml.	0.4 mg./ml.	0.1 mg./ml. +0.05 mg./ml. RN-ase	0.1 mg./ml. +0.05 mg./ml. DNA	0.1 mg./ml. +10 γ/ml. DN-ase
16C	4C			4C			
B			B				
H	EB			B, slow	B	H	H
G		EG	G, slow		H		
P1	LGP	LGP	LGP	G, LGP moribund	LGP	LGP	LGP
P2		P1	P1				
P3	P3		P2	D	P2	LGP, D f.m. present	P2, small
P4	P4	P2	P3		P3		P2
P5	P5 sluggish	P4	D				

* Developmental stages at $t = 20°$ C. See Table I for descriptions.
D = dead embryos.
f. m. = fertilization membrane.

erable to a description (Fig. 6). The "pollen grains" spin around in eccentric orbits unlike the smooth swimming of normal plutei. The developmental arrest was reversible if the embryos were washed repeatedly (after growth in cobalt for 5 hours). At first recovery was slow, but within 48 hours, the embryos appeared similar to normal. Once grossly malformed embryos ("pollen grains") develop and gastrulate, a return to a normal post-gastrulation pattern cannot occur. The addition of DNA, or DN-ase could not inhibit or reverse the cobalt effect to any useful degree (Table IX) even though RNA (10 μg./ml.) and RN-ase (0.05 mg./ml.) slightly ameliorated the effect.

Actinomycin D

The results of growth in actinomycin D with or without other compounds are summarized in Table X. In general, all dose levels, even as low as 3 μg./ml.,

TABLE IX

*The effects of cobalt chloride and cobalt glycine on early development of A. punctulata**

Part A

Control / Sea water	Experimental / Sea water +					
Developmental stages	Cobalt chloride				Cobalt glycine	
	0.02 mg./ml.	0.02 mg./ml.	0.02 mg./ml. +4 γ/ml. DN-ase	0.02 mg./ml. +0.5 mg./ml. RN-ase	0.01 mg./ml.	0.01 mg./ml.
Immersed as E+S	E+S	2C	E+S	E+S	E+S	2C
32C						4C
EB	EB	EB			M	M
H	H	H			EG	EG, H
EG				M		
G	SB					
LGP		LGP	G, LGP	B		
P1	EG	LGP swollen	G, EX	EX	G	G swollen
P2	EX	EX	EX sluggish	EX active		LGP
P3	EX	EX sluggish			G, LGP malformed	LGP, sluggish malformed
P5		EX, highly malformed, spin	EX, moribund	P1 malformed		LGP, malformed

Part B

Control / Sea water	Experimental / Sea water +					
Developmental stages	Cobalt glycine			Cobalt glycine +		
	0.02 mg./ml.	0.2 mg./ml.	1 mg./ml.	0.01 mg./ml. +0.05 mg./ml. DNA	0.02 mg./ml. +8 γ/ml. RNA	0.02 mg./ml. +4 γ/ml. DN-ase
Immersed as E+S	2C	2C	2C	2C	2C	2C
32C		2C, 4C	4C	4C	4C	4C
EB					M	M
H		M	M, D		EG	
EG				M	H	H
G						
LGP	EX	M, B		B	LGP sluggish	G, LGP
P1	LGP	B, M, D		G bloated	LGP malformed	LGP malformed
P2	LGP, EX malformed			EX swollen	LGP malformed	LGP malformed
P3						
P5	LGP malformed			EX swollen	LGP malformed	

* Developmental stages at $t = 20°$ C. See Table I for descriptions.
D = dead.

TABLE X

*The effects of actinomycin D on early development of A. punctulata**

Part A

Control Sea water	Experimental Sea water + actinomycin D							
Immersed as FE	AD 3 μg./ml.	AD 4 μg./ml.	AD 8 μg./ml.	AD 20 μg./ml.	AD 25 μg./ml.	AD 30 μg./ml.	AD 0.75 μg./ml. +5 μg./ml. DNA	AD 4 μg./ml. +0.01 mg./ml. DNA
32C					2C, 4C			
B	B	EB, "lumpy"	EB "lumpy"	M	8C	4C		
H								
SB		B						
LGP	B, G	EG	H, slow		M, D	M, D	EG	G, slow
P1		LGP	G, cell separation					
P2	LGP	LGP, bloated	G, D	M, D				G, moribund
P3	P1	P1, bloated					G	
P4		P1, bloated					LGP	LGP, moribund
P5		P1, have f.m. no arms					D (75 h.) no arms	No skeleton

Part B

Control Sea water	Experimental Sea water + actinomycin D+							
Immersed as FE	AD 8 μg./ml. +0.02 mg./ml. DNA	AD 4 μg./ml. +4 μg./ml. DN-ase	AD 8 μg./ml. +0.1 mg./ml. DN-ase	AD 4 μg./ml. +4 μg./ml. RNA	AD 4 μg./ml. +20 μg./ml. RNA	AD 8 μg./ml. +8 μg./ml. RNA	AD 4 μg./ml. +4 μg./ml. DN-ase	AD 4 μg./ml. +0.05 mg./ml. thymine
32C								
B							B	B
H		M					H	H
SB							SB	SB
LGP	8C, D	B	M, separation of cells	G	SB	M, D	LGP moribund	LGP swollen
P1			M, D		LGP			P1
P2		G, malformed		G, LGP	LPG, swollen			
P3				LGP	LGP, no arms			
P4					LGP			
P5								

* Developmental stages at $t = 20°$ C. See Table I for descriptions.
D = dead embryos.
f.m. = fertilization membrane.

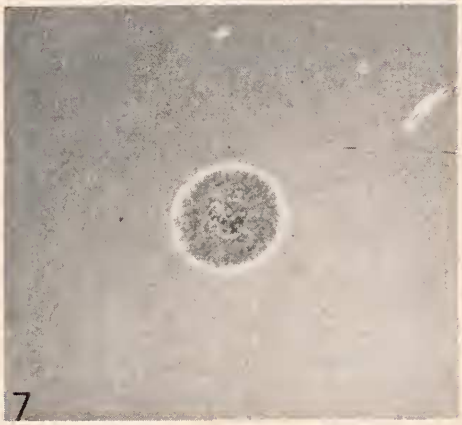
exerted a noticeably retarding effect on development and the retardation was dose-dependent. At 4 μg./ml. the retardation is minimal in the early stages and more striking post-gastrulation in that poorly developed gastrulae cannot differentiate into the pluteus stage. Figure 7 represents a surviving 2-day-old "gastrula" (4 μg./ml.). The bloated larvae (Fig. 8) never develop a skeleton and arms do not develop even after 80 hours survival. With higher concentrations, 8 μg./ml., 20 μg./ml., and 25 μg./ml., the arrest is striking, showing gastrular anomalies (8 μg./ml.), and at 20 μg./ml., 25 μg./ml., and 30 μg./ml., development does not proceed beyond the morula. At 40 μg./ml. most die before the morulae. Doses higher than 40 μg./ml. were not tried because a marginal sensitivity occurred at 2 μg./ml., and a concentration as high as 0.15 mg./ml. existed as a suspension.

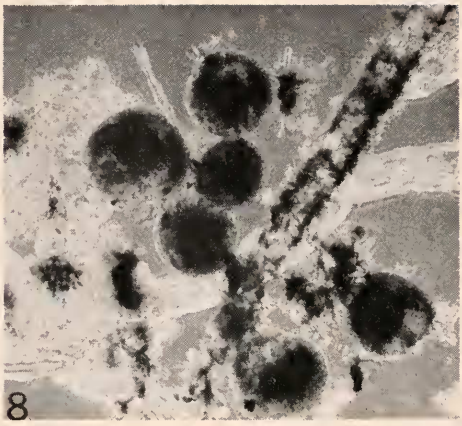
The effects are reversible in so far as survival is concerned, by washing at least within 15 hours, in concentrations up to 8 μg./ml. After that period, washing does not enhance survival. At concentrations of 15 μg./ml. and higher the effects do not appear to be reversible and the embryos die. DN-ase (0.1 mg./ml., 4 μg./ml.) and RNA (4 μg./ml., 8 μg./ml., and 20 μg./ml.) likewise were ineffective in reversing the actinomycin D effect (4 μg./ml., 8 μg./ml.). While RN-ase (0.05 mg./ml.) and thymine (0.05 mg./ml.) appear to ameliorate slightly the actinomycin D (4 μg./ml.) effect in early cleavage stages, the post-gastrulation stages were still retarded. DNA in low concentrations (5 μg./ml. to 0.01 mg./ml.) does not detectably counteract the actinomycin D effect, but at 0.2 mg./ml. does appear to lessen the drastic actinomycin D effect, especially since survival through the pluteus stages occurred. Oddly enough, 0.05 mg./ml. thymine likewise enhanced survival through a young pluteus stage, but again, no arms were formed. The inhibition of formation of arms and skeleton in the pluteus was a characteristic feature even up to three days of development.

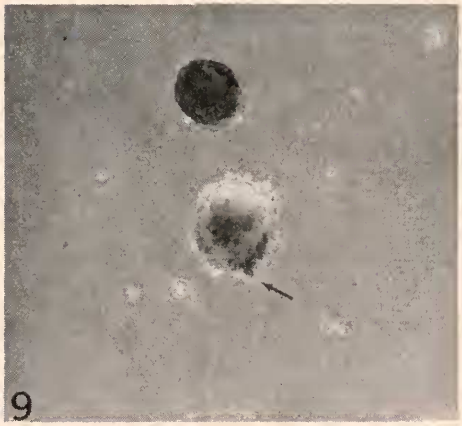
The developmental effects were similar whether eggs and sperm were fertilized in actinomycin D solution or the zygotes were placed into it, and embryos did not show subsequent developmental divergence. If embryos were washed within 9 hours of immersion (4 μg./ml.) they appeared in a better condition than the ones that were unwashed, but the difficulties experienced in gastrulation (abnormalities such as lack of skeleton and arms) were still evident. At 4 μg./ml. there was some evidence of a persistent fertilization membrane about abnormal embryos as late as 80 hours of development. Besides the slowing down of early cleavages, actinomycin D appears to specifically inhibit differentiation of arms and the skeleton. This is especially striking after 75 hours of growth where malformed, arrested late gastrulae eventually die. Figure 8 illustrates these gastrulae, which, while alive, are poorly differentiated, lacking arms, skeleton, and other features of comparable plutei of this age. Another observation bears mention: that is, blastomeres appear to separate at gastrulation with loose cells in the blastocoele (8 μg./ml.). The cleavage disorganization appears quite early, even before the formation of the morula, in that rather "lumpy" or irregularly surfaced embryos develop. This is evident even at a concentration of 4 μg./ml.

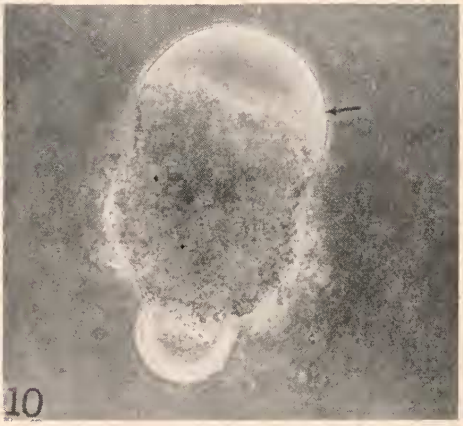
4-nitroquinoline-N-oxide

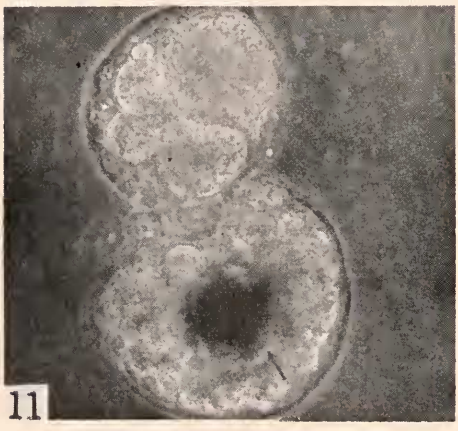
The compound, 4 nitroquinoline-N-oxide (4 NQ), was quite toxic. Eggs were fertilized by normal sperm (presence of fertilization membrane) and immersed in

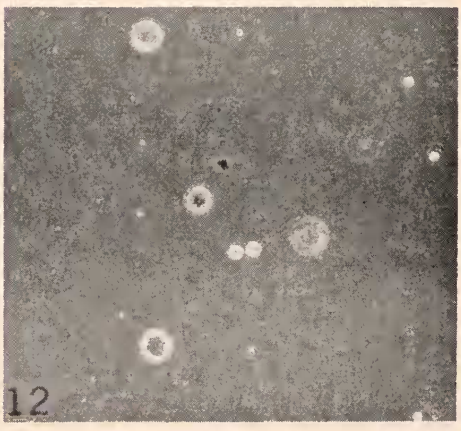












FIGURES 7–12.

a concentration of 0.125 mg./ml., but no cleavage ensued. The cytoplasm of the zygote was shrunken away from the surface of the egg (Fig. 10). Sperm, however, were quite viable in this concentration, showing vigorous activity even after 4½ hours. In 0.0125 mg./ml. an irregular cleavage proceeded along up to the morula stage, with a concomitant separation of blastomeres. Pigment condensation likewise occurred. At half this level, development proceeded to the blastula stage, where it was arrested. Figure 11 illustrates two embryos with irregular-sized blastomeres and pronounced pigment condensation in one. In Figure 12 evidence of complete dispersion of blastomeres is seen after 24 hours.

Coumarin

In coumarin (0.5 mg./ml.) development was slower than normal and never beyond the morula stage. The retardation was more effective if eggs and sperm were immersed separately than after fertilization. If the embryos were washed after five hours development in coumarin (at M stage) some survived for 30 hours. Most of these embryos, however, were deformed spherical masses of cells and spun in eccentric orbits. Figure 13 illustrates embryos in a coumarin solution (0.5 mg./ml.) after 46 hours. Some of the embryos have disaggregated to isolated cells and cell clusters; others are highly irregular but their viability is attested to by their active spinning. By 48 hours most were dead and separation of blastomeres ensued. No gastrulation occurred and no skeletal formation was evident. The block at the blastula stage was striking and irreversible.

Mescaline

A concentration of 1 mg./ml. is lethal, and organisms generally die about gastrulation. Most succumb at the B and M stage. In 0.1 mg./ml. development proceeds but the rate is slower than normal. While the formation of arms in plutei is inhibited, a minuscule skeleton (one-sixth normal size) forms. Although the specimens in Figure 14 appear to be "prisms," they are actually 48 hours old with arm development strikingly inhibited.

RNA (4 μg./ml.) added to the (0.1 mg./ml.) mescaline medium brings about a sharp arrest at gastrulation. Abnormal cell masses due to irregular cleavage result. The 48-hour "plutei" die but bear small spicules. On the other hand, the substitution of DNA (0.05 mg./ml.) terminates development at the blastula stage which developmentally coincides with the prism stage of the control (Table

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FIGURE 7. Arrested embryo in 4 μg./ml. actinomycin D for 48 hours. Note inhibition of gastrulation; phase, 145 ×.

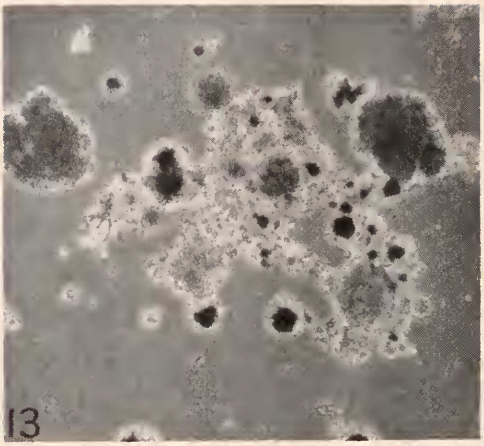
FIGURE 8. Surviving embryos in 4 μg./ml. actinomycin D for 80 hours. Differentiation is halted at gastrulation; phase, 145 ×.

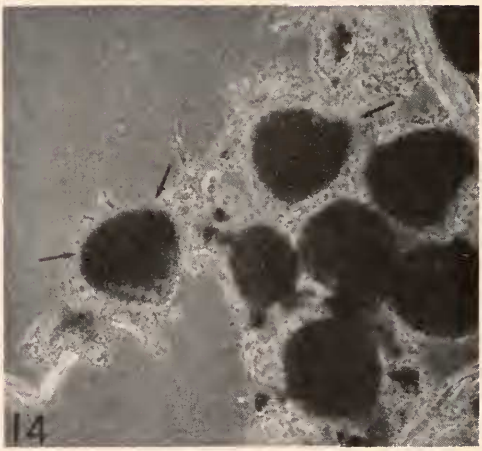
FIGURE 9. With higher levels of actinomycin D (8 μg./ml.) embryos show pronounced irregularity in deposition of surface cells (arrow) and gastrulation is abnormal; phase, 145 ×.

FIGURE 10. Egg of *Arbacia* in 0.125 mg./ml. 4-nitroquinoline N-oxide with drastic shrinkage of cytoplasm (arrow) and cytolysis evident in 15 minutes; phase, 360 ×.

FIGURE 11. Larvae in 12.5 μg./ml. 4-NQ after 8 hours. Note irregular size of blastomeres and pigment condensation (arrow); phase, 360 ×.

FIGURE 12. Complete dispersion of blastomeres after 24 hours in 12.5 μg./ml. 4-NQ; phase, 145 ×.





FIGURES 13–15.

XI). The effects of mescaline are not reversed by placing the embryos in fresh sea water if development has proceeded past gastrulation.

Thalidomide

The effects of thalidomide are charted in Table XII. Because of the poor solubility of thalidomide in aqueous solutions, the concentrations cannot be considered as absolute, but solution in other agents was contraindicated since chemical modifications of the molecule occur. At a level of 0.02 mg./ml. development followed normally; at 0.08 mg./ml. a slowing-down became evident; at 0.1 mg./ml. the retardation was clear. The slower development, nonetheless, did proceed successfully through gastrulation and up to the pluteus stage with some deforma-

TABLE XI

*The effects of mescaline on early development of A. punctulata**

Control / Sea water	Experimental / Sea water + mescaline			
All immersed as FE	0.1 mg./ml.	1 mg./ml.	0.1 mg./ml. +4 γ/ml. RNA	0.1 mg./ml. +0.05 mg./ml. DNA
Developmental stages				
16C	8C	4C		
M	16C			
H				H
SB	H	M		
EG	SB			
G	EG	EB sluggish	G, with abnormal cell masses	D, as B
LGP	G			
P1	LGP, many dead	G	G, static	
P3	P1, sluggish			
P4	P1, no arms but skeleton		D, as G have skeleton, malformed	

* Developmental stages at $t = 20°$ C. See Table I for descriptions.
D = dead embryos.

tions. Figure 15 shows a 46-hour pluteus raised in 0.1 mg./ml. thalidomide. The arms are slightly shorter than those of the normal 2-day-old embryos, and the skeletal formation is poor. Just about the time when elongation of the arms of the pluteus occurred (stage P4–P5), the sluggish plutei died, many remaining within an intact fertilization membrane. At 1 mg./ml. the same pattern evolved but the retardation was greater. Of considerable interest is the persistence of

FIGURE 13. Embryos although alive in coumarin (0.5 mg./ml.) after 48 hours consist of misshapen balls of cells with much separation of blastomeres; phase, 145 ×.

FIGURE 14. Specimens raised 48 hours in mescaline (0.1 mg./ml.). Arm development (arrows) arrested; phase, 145 ×.

FIGURE 15. A pluteus larva after 48 hours in thalidomide (0.1 mg./ml.). Morphology essentially normal except for slightly shorter arms and poorer skeletal formation than the control; phase, 180 ×.

TABLE XII

*The effects of thalidomide on early development of A. punctulata**

Control: Sea water	Experimental: Sea water + thalidomide					
All immersed as 2C	0.02 mg./ml.	0.1 mg./ml.	0.5 mg./ml.	1 mg./ml.	2.5 mg./ml.	0.5 mg./ml. +0.04 mg./ml. RNA
Developmental stages						
16C		4C, 8C		2C, 4C		
EB						
SB		M	EB	M		
G	G				32C	EG
LGP		G	EG		D	
P1	P1		LGP	G		
P2		P1		EG		
P3	P2, P3		P1	M, G		LGP, P1
P4	P4	P2, moribund	P3	LGP		P1, P2 sluggish
P5+	P5+	P3, D	P4, small	P1, inactive		

Control: Sea water	Experimental: Sea water + thalidomide				
All immersed as 2C	1 mg./ml. +0.04 mg./ml. RNA	1 mg./ml. +0.05 mg./ml. DNA	1 mg./ml. +10 μg./ml. DN-ase	1 mg./ml. +0.05 mg./ml. DNA +10 μg./ml. RNA	0.5 mg./ml. +0.1 mg./ml. cobalt glycine
Developmental stages					
16C					
EB		4C, D	M, D	2C, D	
SB					
G					
LGP	EG				SB, LGP
P1					
P2					
P3	G, D				SB, LGP moribund
P4					P1
P5+					D

* Developmental stages at $t = 20°$ C. See Table I for descriptions.
D = dead.

the fertilization membrane about the plutei, which indicates a fault in the hatching enzyme. Greater doses were lethal (Table XII).

The thalidomide effect is irreversible in that even if the zygotes are washed free from the compound, the retardation persists. Moreover, it appears that the zygote is more adversely affected than if eggs and sperm are immersed separately prior to fertilization. Skeletal formation generally occurred normally. The addition of other agents (RNA, cobalt glycine, DNA, DN-ase, DNA + RNA) greatly enhanced the retardation or lethal effects of thalidomide.

Discussion

General

The sea urchin egg and zygote, especially of the genus *Arbacia,* have been for many years the favorite material for the investigation of early morphogenesis. Within the last decade biochemical studies on the metabolism of nucleic acids, proteins, and carbohydrates have increased enormously. Since competent reviews and papers in various areas are available (*vide:* Gustafson, 1954; Costello, 1955; Ranzi, 1957, 1962; Deuchar, 1962; Guidice *et al.,* 1962; Karnofsky and Simmel, 1963; Gross, 1964; Lallier, 1964a; Monroy and Maggio, 1964, Brachet, 1965a, 1965b; *inter alii*), only particularly relevant papers will be cited without attempting to identify *seriatim* all the excellent work in this field.

The problem of comparing data not only from one experiment to another, but from one investigation to another has become a very real one. Comparisons are hindered because of variations during the experiments *per se.* As Harvey (1956) states: "There is a slight variation in cleavage times in different batches of eggs and considerable variation at different times of year, irrespective of temperature . . . there is a greater variation in the later stages of development, *e.g.,* time of hatching" (p. 97). Thus expressing development as being "5½ hours post-fertilization" or "20 hours development" as is often done, is uninformative. It is the precise developmental *stage* which needs to be identified, the stage at which critical and profound biochemical events come into play.

Accordingly, the symbols and developmental stages listed in Table I have been selected as being the most diagnostic—from the immature oocyte to the long-armed, non-feeding pluteus. The need for clarity, brevity, and accuracy in staging has been considered, as well as time and temperature factors. Although this series (Table I) refers specifically to *Arbacia punctulata,* the designated *stages* may be used as a guide for level of development in other species or genera in the echinoids, especially when data are compared. Hopefully, similar studies will be made on pre- and post-metamorphic echinoderms in order to establish and tabulate morphological criteria of the stage of development. The same cautions would apply as in the formulation of Table I.

The prime advantage of the present multi-pronged approach, that is, being able to compare the effects of a wide spectrum of agents, is that all departures from the normal developmental pattern can be readily compared and non-specific effects can be assessed as well as highly specific effects. The present morphological study may serve to supplement previous studies. Attempts were made to follow development through to the feeding pluteus larva, the embryo being considered as a total system.

Alcohol

Since actinomycin D (0.5 μg./ml.) had to be solubilized in 95% ethanol, the question of the effect of a weak alcohol solution on growth had to be rechecked. Over 50 years ago Lillie (1914) reported that 5% ethanol (0.87 *M*) acted as an anaesthetic on sea urchins in that there was a reversible arrest of cleavage. Later Blumenthal (1928) confirmed the anaesthetic effects of alcohol on sea urchin zygotes. Waterman's (1936) data indicated that ethyl alcohol up to 0.9% had

no effect and retardation occurred with a concentration of 1 to 2%. A reversible effect was possible with concentrations up to 4.4% but 5% was lethal. Exogastrulae developed when concentrations ranged from 0.9 to 1.3%, but this was not observed in our studies.

The concentration of alcohol in the present experiments (actinomycin) was always well below 0.9% and, therefore, did not affect cleavage. Our experiments, likewise, confirmed the lethal effect of 5% ethanol and indicated that the effects of methanol (5%) were similar. The lethal higher concentrations of alcohol probably operate as lipid solvents and denaturating agents. The swelling, doubling in volume, in ethanol and methanol reported by Stewart (1931), was not evident.

Enzymes

Enzymatic dissociation of multicellular tissues is by now a routine procedure but it was deemed necessary to evaluate the effects of the commonly used proteolytic agents on sea urchin embryogenesis, *per se*.

Northrup (1947) demonstrated the fact that proteolytic enzymes (0.1 to 1 mg./ml.) did not lyse mature protein in that *Arbacia* developed normally in ficin and trypsin, with separation of blastomeres occurring only in papain. He emphasized the fact that trypsin did not affect living cells with native protein and attacked only denatured protein. Moore (1949), on the other hand, observed trypsin lysing the fertilization membrane and effecting cell disaggregation. Later, Moore (1952) followed the gastrulation of *Dendraster* in trypsin (1 mg./ml.). Embryos with elevated fertilization membranes showed a lysis of this structure within 5 to 10 minutes. The denuded embryos generally lost blastomeres at invagination, thus larvae developed as malformed plutei with defective skeletons. Moore's (1951) interpretation was that blastomeres released tryptic inhibitors.

The present results confirm the usefulness of trypsin as a dispersing agent for blastomeres and fertilization membranes. It must be used at less than 0.5 mg./ml. and be washed free from the cultures within one hour and certainly before 4 hours, to prevent death of the embryos. The harmful effects may be due to the penetration of trypsin into cells as has been shown by Weiss (1958), who determined that a 20% loss in the dry mass of sarcoma 37 cells occurred as a result of trypsin treatment.

The data of Jensen (1950) and Hörstadius (1953) indicate that ficin and trypsin have similar proteolytic activity. The action of papain is somewhat similar. In 1947 Northrup described the separation of *Arbacia* blastomeres in papain. We confirmed this observation and also observed the dissolution of the fertilization membrane. The lysis, however, took longer than in trypsin and undesirable cytologic effects also resulted (Mateyko, 1965). In general, *Arbacia* embryos developed in papain solutions but the effects were more drastic than in trypsin, and for survival a thorough washing was needed. Discrepancies in the literature as to the efficacy of trypsin in lysing the fertilization membrane or effecting cell dispersal thus may be partly explained by the different dose levels, purity, time, and duration of exposure to the enzyme.

Berg (1958) achieved dissolution of the fertilization membrane of *Dendraster* by 0.1% protease (non-specified) but needed 5% to get disaggregated blastomeres. In 1959 Nomoto and Narahashi purified a protease elaborated by *Streptomyces*

griseus which they called Streptomyces protease G. According to Hiramatsu and Ouchi (1963) this enzyme, now called Pronase P, had four active proteolytic fractions. Mintz (1962) reported that 0.5% pronase could be used to safely remove the zona pellucida of mammalian egg cells and Gwatkin and Thomson (1964) used it to disperse both embryonic and adult cells of mice. Later it was shown that pronase was a desirable cell-dissociating enzyme for frog renal tissues (Mateyko and Kopac, 1965).

The effects of pronase on sea urchin development were of interest. Development proceeded normally through the pluteus in 2.5 mg./ml. solutions but when the fertilization membrane lysed, as it did frequently, separation of blastomeres occurred. When eggs were immersed in pronase (0.25 mg./ml.) prior to fertilization, a reduction in cortical consistency was noted and cytolysis followed. Cortical changes elicited by trypsin pretreatment are described by Runnström (1948). Thus the fertilized egg is resistant to the proteolytic action of pronase. Sperm cells, on the other hand, remained motile for several hours and capable of fertilization at the same concentration that caused extensive damage to ova. This variety of response, however, is not unusual when the literature is examined. To reiterate briefly, Runnström and Krizat's (1962) data demonstrated that trypsin elicited cytoplasmic changes in consistency which affected the adherence or elevation of the fertilization membrane. This may also be true for pronase. In addition, when lysis of the membrane occurs, separation of blastomeres is not an unforeseen event. When it is intact, normal plutei develop in pronase solutions. Moore's (1949) data indicate that the time and dose factors for proteolytic action are of importance in that trypsin (and most likely pronase, too) may eventually digest the hyaline layer, thus facilitating separation of cells.

The fact that sperm were so little affected by pronase suggests that pronase affects a cytoplasmic target. Of all the enzymes studied, pronase is the only one in which normal growth and survival continued for two days without washing.

Although sodium taurocholate is not an enzyme, it is a surface-acting agent. Moreover, Rojas (1965) states that the "cell surface protein integrity" generally impaired by proteolytic enzymes is essential for the maintenance of the properties and physiology of the cell membrane. Accordingly, it is considered along with the proteases. Sodium taurocholate, because of its well-known action on lipids of cell membranes, usually brings about lysis of cells. It causes gross changes in the surface structure of erythrocytes (Ponder, 1961) and may do this by enhancing lipolytic action by uncoupling lipase diglyceride complexes (Fritz and Melius, 1963). It may be, in general, an activator of lipases.

There is only one paper, that of Genther and Schmidt (1931) on the effect of this substance on echinoderms. They conjectured that there might be a growth stimulant effect on *Arbacia*. With a mixture of taurocholate and glycocholate they noted that the rate of division and retardation of development were proportional to the concentration of the bile salts.

Our results show that in low concentrations sodium taurocholate (0.2 mg./ml.) did not enhance or retard embryogenesis, while in stronger solutions there was a cleavage arrest and death at the blastula stage with pronounced cellular swelling and erosion of the surface of cells. Similar surface denaturation of frog renal tumor cells occurred after exposure to sodium taurocholate. Even concentrations

as low as 0.0003 *M* brought about cytoplasmic alterations (Mateyko and Kopac, 1965).

Allied studies bear mention. Gustafson and Sävhagen (1949) noted that anionic detergents by virtue of their surfactant properties induced radialization in sea urchins. Hagström and Holman (1965) concluded from their work on the effect of free fatty acids on the fertilization of sea urchin eggs that the promoting or negative effects are structure-dependent. The retarding effects of oxidized lipoic acid, especially after blastulation and at gastrulation of sea urchins, are described by Wolfson and Fry (1965).

DNA

The more recent accounts of DNA levels in the echinoid eggs are those of Hoff-Jørgensen (1954), Agrell (1958b), Nigon *et al.* (1963), Troll *et al.* (1964), and Pikó and Tyler (1965), which concur that the cytoplasm contains sizably more DNA than the nucleus, and that the deoxyriboside material is transformed into nuclear DNA during cleavage. In their review Monroy and Maggio (1964) summarize the wealth of biochemical evidence which indicates that the synthesis of DNA in the sea urchin embryo begins quite early after fertilization and may draw upon the precursors resident in the cytoplasm of the mature egg.

Although there is no conclusive evidence whether the intact macromolecule of DNA or one of its degradation products enters a cell which is exposed to a medium containing exogenous DNA, there is no doubt that it does exert a cellular effect. Gartler (1959, 1960), using labeled DNA and cells in tissue culture, showed that a small amount of intact DNA was taken up while some was degraded. The uptake of large fragments of DNA under *in vitro* conditions was confirmed by Chorazy *et al.* (1960), Bensch and King (1961) and Schimizu *et al.* (1962) using Ehrlich ascites, strain L, and Ehrlich ascites cells, respectively. To illustrate further, the penetrability of DNA (2 to 100 mg./ml.) into HeLa cells was demonstrated by Borenfreund and Bendich (1961). H^3DNA, DN-ase-labile, localized in nuclei. Gosse *et al.* (1965), however, do point out that tissue DN-ases degrade exogenous DNA which is injected into mammals. Ledoux (1965) reviews the area of the uptake and subsequent metabolism of DNA by living cells.

By far one of the most stimulating papers is that of Mazia (1949). He noted that the occurrence of cytoplasmic DNA and DN-ase was far above the amount borne by the sperm cell and suggested that the excess DNA was non-genetic. Using *Asterias forbesii* as the developing form, Mazia observed that embryos were stopped at the gastrula stage with homologous DNA (sperm) but not from a heterologous sperm source (*viz. Arbacia*). Thus development was normal in foreign DNA. Hörstadius *et al.* (1954) expanded this facet of work. They extracted DNA from several echinoids and found a variety of responses. DNA from the same species, *Paracentrotus lividus*, did not arrest development but that from *Echinocardium cordatum* was more injurious, etc.

Eggs of *Paracentrotus* developed in DNA solution but many died; in 0.25%, the pluteus stage was reached; in 0.5% the gastrula-prism stage was reached. Moreover, animal halves were animalized if raised in DNA solution. Microinjection of DNA solutions into the egg cytoplasm was also performed. If the

droplet did not mix with the cytoplasm, the eggs were fertilizable, development proceeded normally, and the droplet was then extruded.

If the droplet diffused through the cytoplasm, an activation occurred which was followed by cytolysis.

Exogenous DNA has been added to sea urchin embryos as a means of counteracting the developmental arrest produced by actinomycin D (Lallier, 1963a, 1963b). The interaction is discussed in the section on the actinomycin effect. DNA, when added to *Arbacia* embryos at concentrations below 0.05 mg./ml., did not alter appreciably the rate of development or morphological patterns, but at double this level, the growth retardation was evident. In our experiments, *Arbacia* embryos were sluggish at the prism stage with some deaths at the pluteus at 0.1 mg./ml.

Although skeletal differentiation occurred, often the gastrular stage was maintained. Thus the mesodermal derivatives are not inhibited although there is some disruption in morphogenesis. This may be possibly a facet of "animalization" according to the descriptions of this phenomenon by Hörstadius (1949).

DN-ase

The direct *in vivo* or *in vitro* effect of exogenous DN-ase has not been as popular a study as that of RN-ase. A comparison of available data is not satisfactory because of the variety of responses elicited. The effect of DN-ase on cellular DNA of living cells was reported by Kaufmann and Das (1955) who found no growth interference with the addition of DN-ase to root tips, and suggested that the molecule was probably unable to penetrate. The injection of DN-ase did not alter the Feulgen reaction or basophilia in amoeba (Brachet, 1959). The addition of acid DN-ase to embryonic chick cells in culture elicited some mitotic inhibition and a mitochondrial modification (Chèvremont and Chèvremont, 1957). De Lamirande (1961) observed a disappearance of DNA from tumor cell nuclei upon injection of DN-ase to mice with ascites. The incorporation of H^3-thymidine and oxyuridine was depressed only slightly by the addition of DN-ase to HeLa cells in culture (Feinendegen *et al.*, 1961).

In 1948 Mazia *et al.* demonstrated that most of the DN-ase of the unfertilized *A. punctulata* eggs was cytoplasmic. He also reported that cytoplasmic DNA and DN-ase were far above that borne by the sperm cell (Mazia, 1949). Moreover, nuclei isolated from cleavage stages had high levels of DNA polymerase (Mazia and Hinegardner, 1963).

Since our *Arbacia* raised in sea water containing DN-ase showed a retardation of growth and activity, it is clear that the commercially available macromolecule is effective. The effect of the exogenous DN-ase appears to be a non-specific one on *A. punctulata,* in that at high levels animals die while in lower levels (0.05 mg./ml.) only the activity but not morphogenesis is curtailed. A block in embryogenesis by the exhaustion of DNA, as suggested by Moore (1955, 1958), which occurs to a certain extent at the blastula, could possibly be accelerated by DN-ase.

RNA

Since it was conjectured that the inhibiting role of cobalt might be reversed by RNA, the effects of both RNA and RN-ase were determined. One can only em-

phasize the complexities of RNA metabolism in the development of the sea urchin since a full discussion is beyond the scope of this paper (*vide:* Brachet *et al.,* 1963; Comb and Brown, 1964; Gross and Cousineau, 1964; Gross *et al.,* 1964, 1965; Hultin, 1964; Brachet, 1965a; Comb *et al.,* 1965; and Whiteley *et al.,* 1966).

The relatively large stores of RNA that are present in the fertilized egg (Immers, 1960; Pasteels *et al.,* 1958) and the fact that a limited RNA synthesis occurs from fertilization to blastulation (*vide supra* and review by Monroy and Maggio, 1964) make it unlikely that exogenous RNA, especially in small quantities, can exert any striking effects on developing *Arbacia* zygotes.

In 1957 Niu and Douglas added RNA extracted from *Raia erinacea* to 2-cell embryos of *Styela* and *Arbacia* and found that the rate of development increased with the addition of 5 μg. to 100 μg./ml. for *Styela* and 4 to 16 μg./ml. for *Arbacia.* Our results are in general agreement with these observations, the acceleration being evident at 10 μg./ml. and a definite retardation at 0.1 mg./ml. Developmental aberrations were not produced. The effect of RNA on *Paracentrotus* is reported by Lallier (1964b). The duration of exposure was 30 minutes in 1 mg./ml. RNA is described as inducing radial symmetry and augmenting animalizing effects. We found, however, that the solubility of RNA, unlike RN-ase, was poor at 1 mg./ml. and in order to have an effective solution, a lower concentration had to be used. Very recently Lallier (1966) reported that sRNA (1 mg./ml.) even when subjected to prolonged (15 hours) alkaline hydrolysis exerted a radializing effect on *Paracentrotus lividus* embryos. Cytidylic acid also had this effect but not hydrolyzed thymus RNA.

In a study of the effect of RNA on development of *Strongylocentrotus purpuratus,* Whiteley *et al.* (1965) reported that RNA competes in the binding with DNA, and that RNA from ova and blastulae, and hatching embryos competed less strongly than pluteus RNA. This RNA is mRNA.

In addition, Lansing and Rosenthal's (1952) observation that RNA is present at the cell surface of *Arbacia,* and may participate in ionic transport, is of interest.

It is generally accepted as a dictum that organized nucleoli represent a major repository of nuclear RNA unless mitoses are too rapid, as during embryogenesis, to permit the assembly of RNA into this organelle. In amphibian development Brachet (1964) and Brown and Littna (1964) demonstrated that the evolution of discrete nucleoli at gastrulation coincided with the synthesis of ribosomal RNA.

It is surprising that in the wealth of work on echinoderm development no definitive study has been made on nuclear morphology, specifically on the nucleolus during embryogenesis. In *Paracentrotus miliaris,* Agrell (1958a) pointed out that no nucleoli were noticed up to the 32-cell stage. The chemical study of Cowden and Lehman (1963) on the development of *Lytechinus verigatus* (*sic*) comments on the lack of nucleoli at the fourth cleavage and even in the mesenchyme blastula, and reports that nucleoli appear for the first time at the early gastrula stage. There is, however, more cytoplasmic RNA after the fourth cleavage. Harris (1961) in an electron microscopic study of mitosis (16 to 32 cells) describes in the interphasic blastomere nuclei of *Strongylocentrotus* "a number of dense ovoid bodies of unknown significance." Thus a systematic cytological analysis of nucleoli, documented by photographs, from the 2-cell stage through gastrulation has to my knowledge not been done for any echinoid. One may conjecture, nonetheless, that

during embryogenesis RNA synthesis does occur even before visibly organized nucleoli appear.

RN-ase

That ribonuclease can penetrate living cells and elicit a diminished cytoplasmic basophilia is now well-established (Brachet, 1955a, 1955b; Firket *et al.*, 1955). To illustrate, in *Asterias glacialis* oocytes, a diminution in basophilia was noticed after RN-ase (1 mg./ml.). Brachet (1955b) thus concluded that RN-ase penetrated living cells and thus brought about a cessation of mitosis and an inhibition of incorporation of amino acids into proteins.

The literature describing the effect of RN-ase on echinoderm development is relatively sparse, but agrees in general that cleavage arrest is a principal phenomenon. Leone's (1960) studies on *Arbacia punctulata* development in RN-ase (crystalline, 1%) indicated that the most sensitive stage was the fertilized egg, only 20% of the embryos reaching the pluteus. In our experience this was a toxic dose. Ledoux and Metz (1960) also noted an inhibition of sea urchin cleavage by RN-ase but their concentrations were less. The degree of inhibition depended not only on the dose but on the commercial source of enzyme. In *Lytechinus variegatus* but not especially *Arbacia punctulata,* there was a "collapse and partial lysis" of the fertilization membrane. RN-ase at 1.5×10^{-4} *M* definitely exerted a cleavage block.

In our experiments on *Arbacia,* below 0.1 mg./ml. RN-ase, development appeared to run parallel to normal; between 0.1 to 0.12 mg./ml. a slight developmental retardation was produced but normally differentiated plutei resulted. Thus no striking morphogenetic changes ensued when non-toxic doses were applied.

The possible surface effects on RN-ase should be considered. Lansing and Rosenthal (1952) described RNA at the cell surface of *Arbacia.* RN-ase could attack this region.

Cormack (1966) demonstrated that RN-ase (1.5×10^{-3} *M*) conjugated with fluorescein isothyocyanate 3 minutes after fertilization, blocked the cleavage of 90% of *L. variegatus* and *A. punctulata* while the rest were arrested at 2- or 4-cell stage. This is, however, a high dose. The fluorescein-labeled RN-ase was absorbed to the fertilization membrane and hyaline layer, but was not detectable within the egg. However, during development from the blastula to the pluteus, the labeled RN-ase was incorporated into cytoplasmic droplets by cells of the digestive tract. The reversibility by washing also pointed to a possible surface effect of RN-ase.

Interpreting these data, it is clear that small amounts of exogenous RN-ase do not alter appreciatively the development of the echinoid zygote. Larger, but non-toxic, amounts slow down the embryogenesis but do not shut down mitosis or bring about any changes in the direction of morphogenesis. Larger amounts of RN-ase, which are cytotoxic, may effect this *via* a massive degradation of all types of cellular RNA and thus a disorganization of RNA or even protein metabolism. A surface effect on ionic transport is also to be considered.

Thymine

One may conjecture that thymine may function as a growth factor by virtue of its conversion to 5-hydroxy-methyluracil or to thymidine (Abbott *et al.,* 1964),

and subsequent utilization as a DNA precursor, but experimental evidence shows that it is generally inhibitory. It slows the rate of cell division in onion root tips, (Deysson, 1954), and may inhibit the timing of mitosis as adenine does (Biesele *et al.,* 1952; Brachet, 1959).

According to Brachet (1959) purines and pyrimidines inhibit growth and regeneration in *Acetabularia,* the pyrimidines being less inhibitory. In general, sea urchin embryos can utilize nucleosides in biosynthetic reactions (Nemer, 1962). The morphogenetic effects of some nucleotide metabolites on sea urchin development have been assayed by Markman (1964). Generally, in low concentrations a slight acceleration of development may occur. But cleavage retardation by pyrimidine derivatives has been reported by Stearns *et al.* (1962) for *Strongylocentrotus.*

In sea urchin development, low concentrations of thymine are mildly retarding to growth, but development proceeds normally. The higher concentrations appear to be particularly inhibitory at the transition from blastula to gastrula. Although the cleavage block is striking in thymine solutions (0.1 to 0.4 mg./ml.) and not alleviated by RN-ase or DN-ase, the binding of thymine must be weak since recovery is rapid upon removal of embryos to fresh sea water. The very striking retardation of differentiation and growth elicited by DNA plus thymine may be explained perhaps by the toxicity attributed to pyrimidine bases (Biesele *et al.,* 1952) and nucleic acids (Smith, 1964). It should be mentioned, however, that the block to the development of *Paracentrotus* at 16 to 32 cells by 5-fluorouracil was reversed by thymine and thymidine. According to Lallier (1965) this suggests that the pyrimidine analogue interferes with the elaboration of thymine necessary for the synthesis of DNA.

Cobalt

In 1923 Hoadley made a study of the effect of metal salts, including cobalt chloride, on the fertilization reaction in *Arbacia,* and reported a cleavage toxicity for cobalt in 1:100 concentration. In the present experiments, the retarding effects on cleavage are reversible if the concentrations are low (0.01 mg./ml.) and the treatment does not extend beyond 5 hours. At higher concentrations (to 0.02 mg./ml.) the drastic exogastrulation is irreversible. Rulon's (1956) study on sand dollar embryos revealed that cobalt brought about developmental modifications at low concentrations and inhibited cleavage and elicited exogastrulation at higher concentrations. More recent work confirms these inhibitions of development by cobalt (Mateyko, 1961). Our data have shown that cleavage, *per se,* is not blocked by cobalt in low concentration, but that embryogenesis proceeds at a slower rate.

Since the cobalt effect is produced with equal facility by cobalt chloride and cobalt glycine, the effectiveness of the cobalt ion, rather than the glycine moiety, is clear. According to Mastrangelo (1966) glycine does not have any effect on the development of various echinoids.

Rulon (1956) had noted that there was a lack of cohesiveness in the cobalt-treated *Dendraster* embryos. A tendency toward cellular disaggregation was also evident in *Arbacia* larvae, but the phenomenon was never very striking. Our results show that at gastrulation, the morphogenetic directions are misrouted, and

irreversibly so. It thus appears that the unique cobalt effect is locked in at gastrulation when a dramatic change in form, "the pollen grain" exogastrulae are produced. This alteration is so striking and so characteristic that it may be described as the specific cobalt effect. Among the other structural effects were: cleavage retardation and irregular deposition of cells, polar elongation, exogastrulation, inhibition of proper differentiation of spicules, and swelling ("bloating"). These aberrations are similar not only to those reported by Rulon (1956) for *Dendraster*, but also closely resemble the original lithium effect of Herbst (1892). Likewise, the cobalt-induced exogastrulae are closely identifiable with the Hörstadius (1949) figures on vegetalization brought about by lithium. In some instances, *Arbacia* embryos showed a slight degree of radialization, the phenomenon being characteristic of weak animalizing agents.

It has been known since the publication of Lindahl's (1936) monumental work, that glycolysis and respiration are inhibited in echinoids by lithium. The paper, although 30 years old, bears careful re-reading. In Lallier's (1964a) survey on the lithium effects in echinoderms, it is clear that many aspects of cell metabolism are affected by lithium—respiration, glycolysis, ATP synthesis, nucleoside metabolism, etc. Comparable work on cobalt is sparse.

The metabolic target of cobalt is difficult to pinpoint because of the many intracellular effects. To specify, Laskowski (1961) points out that DN-ase I requires cobaltous ions for activity whereas soybean DN-ase is inhibited by cobalt. It has long been evident that cobalt is inhibiting to several enzymes. Levy *et al.* (1950) demonstrated the inhibition by cobalt of succinoxidase, choline oxidase, cytochrome oxidase, catalase, and choline dehydrogenase and succinic dehydrogenase in mammalian tissues. Cobalt also acts as an inhibitor of purified α-oxoglutarate dehydrogenase *in vitro* (Webb, 1964).

A generalized biological action of cobalt was interpreted by Webb (1962) to be an inactivation of systems that are dependent upon the reversible interconversion of lipoic acid and dihydrolipoic acid. Rulon (1963) suggested that cobalt ions became attached to SH groups at the surface of *Strongylocentrotus* eggs. In their studies of cellular morphogenesis in *Psammechinus*, Gustafson and Wolpert (1962) account for structural changes in general by a "change in contact between individual cells and between cells and the hyaline membrane." Whether exogastrulation is attributable to cobalt-induced metabolic disturbances directly, or is due to a secondary change in the surface properties of cells is unclear. Weiss (1950) has stressed the importance of the latter in differentiating systems where exogastrulation occurs.

Lithium affects ribonuclease (Bigelow and Geschwind, 1961); cobalt inhibits bacterial ribonuclease (Muira and Nakamura, 1951). It is not evident, however, whether the inhibiting effect of lithium bromide on ribonuclease can in any way be compared to the inhibiting effects of cobalt chloride on ribonuclease.

The observations that cobalt inhibited bacterial RN-ase (Muira and Nakamura, 1951), that it produced persistent nucleoli in tissue culture (Heath, 1954), that it inhibited intracellular RN-ase in *Tetrahymena* as well as crystalline RN-ase (Roth, 1956), that it brought about formation of extra micronucleoli in *Vicia faba* (Komczynski *et al.*, 1963) all suggested that cobalt was involved in the metabolism of ribonucleic acid. In our experiments, the addition of RN-ase, which in itself

is growth-retarding in concentrations greater than 0.05 mg./ml., produced more active, but still malformed exogastrulae. The ameliorating effects of RNA were also quite slight, although Lallier (1946b) found that lithium-vegetalized sea urchins were "equalized" by the addition of RNA. He indicates that soluble RNA influences morphogenesis in *Paracentrotus* by regulating protein synthesis. Possibly when net nuclear RNA synthesis is established at gastrulation and major morphogenetic events occur (Brachet, 1965a, 1965b) the interference of the cobalt ions in cell metabolism seems evident. Moreover, the addition of DNA and DN-ase did not reverse the effect. In fact, the addition of DNA to cobalt-treated embryos arrested growth of *Arbacia* even earlier.

One can speculate, then, if nuclei of the sea urchin are irreversibly differentiated at gastrulation, the cobalt effect may be stabilized in these differentiated nuclei.

Actinomycin D

Muira (1953) found that actinomycin A at 100 mg./ml. slightly retarded the rate of cleavage in *Arbacia punctulata*. Later Wolsky and Wolsky (1961) observed if fertilization occurred in actinomycin (25 μg./ml.), there was a retardation, anomalous growth, and death before gastrulation. Our data showed that 5 μg./ml. actinomycin D was effective in arresting most embryos at the blastula (Mateyko, 1965). A series of more detailed papers on echinoid morphogenesis followed which generally concurred that actinomycin D caused a dose-dependent inhibition of larval cleavage and differentiation (Brachet *et al.*, 1963; Lallier, 1963a, 1963b; Markman and Runnström, 1963; Gross and Cousineau, 1964; Markman, 1964; Guidice and Hörstadius, 1965). The present morphological results, while agreeing essentially, do show some departures that bear discussion. These involve the effective dose level for retardation, the specific morphological effects and the stage of blocking, and attempts at amelioration by other molecules.

The sample of actinomycin D (provided by Merck, Sharpe, and Dohme) has a low aqueous solubility, especially in sea water. Because of this property, generally solvents such as acetone, ethanol, or propylene glycol, etc., have been used for the initial preparation of stock solutions. Although Gross and Cousineau (1964) made a stock solution of 246 μg./ml. in sea water, our sample on standing showed particles in suspension at 0.15 mg./ml. Thus our stock solutions were made at lower concentrations in distilled water (100 μg./ml.) and in 95% ethanol (0.5 mg./ml.) to insure non-precipitating solutions.

In our experiments the developing *Arbacia* embryos were susceptible to actinomycin D at quite low levels (2 to 3 μg./ml.) and it is our thesis that the precipitability of actinomycin D in sea water may be a factor. That actinomycin D is biochemically active at concentrations much lower than those that bring about developmental aberrations is presented by Gross and Cousineau (1964) who indicated that a concentration as low as 1.4 μg./ml. was effective in inhibiting the uptake of C^{14}-uracil by 64% (the full effect of 94% inhibition occurring at 24 μg./ml.). Our studies on the actinomycin effect were designed, therefore, to check the "marginal" concentration for response such as has been described by Reich *et al.* (1962) for HeLa cells.

The specific morphological effects and stage of blocking again are of interest in that lower concentrations of the substance than previously reported have been effective, and development has been followed for several days or until the embryos succumbed.

At 2 μg./ml., a marginal dose, there was an inhibition in that *Arbacia* embryos were slower cleaving and thus morphologically younger than the controls, and while they did reach a pluteus stage, the growth of the arms was retarded. At levels below 3 μg./ml. cleavage was retarded but proceeded through gastrulation, and some abnormalities occurred during this transition. The striking effect was noted at 4 μg./ml. Up to the point of gastrulation, cleavage proceeded slowly, but gastrulation *per se* was abnormal and further differentiation to the pluteus stage was blocked in that skeletal and arm formation was inhibited. Lallier's (1963a, 1963b) data on *Paracentrotus lividus* grown in an actinomycin concentration of 5 μg./ml. are in agreement in that asymmetric gastrulae were produced without spicules. These recovered when placed into fresh sea water.

Gross and Cousineau (1964) reported that between 6 to 20 μg./ml. actinomycin D, cleavages were faster and at 6 μg./ml. were at the same rate as the controls, but even at this level irregular blastulae were produced. Some failures of gastrulation occurred even at 1.4 μg./ml. Our experiments demonstrated that even at 3 μg./ml., the retardation in early cleavage was indisputable and no enhancement of division was found even at the 2 to 4 μg./ml. level. Disorganization in the regularity of cleavage appeared quite early even at 4 μg./ml. in that there was an irregular disposition of surface cells even before the morula stage so that a "lumpy" surface was characteristic.

The effects of low levels of actinomycin D, however, are even more apparent when embryos are maintained beyond 24 hours. We found that while the early cleavage stages showed retardation, the older organisms were even more striking in that bloated embryos survived for 5 days, spinning about but never producing arms, skeleton, or other evidence of further differentiation.

In the present experiments with 20 to 30 μg./ml. of actinomycin D, our *Arbacia* embryos died at the morula stage, while at 40 μg./ml. they did not reach the sixth cleavage. Even at 8 μg./ml., the attempt at gastrulation often failed. The fact that Gross and Cousineau (1964) raised embryos, albeit with some abnormalities, in concentrations of 65 μg. to 120 μg./ml. may be explained by a difference in the solubility of the samples.

We are in agreement with Lallier (1963b) in that a dose-dependent inhibition of development is present and that a fresh sea water milieu enhances survival if the concentration of the antibiotic does not exceed 10 μg./ml. But even at this level, abnormalities of gastrulation occurred. Gross and Cousineau (1964) report that at 65 μg./ml. the embryos are inert multicellular masses compared to controls which are gastrulae. Our results indicate that at 20 μg./ml. development could not proceed beyond the morula stage. In order to get the morphogenetic block at the blastula, concentrations no greater than 8 μg./ml. had to be used. Under these conditions, survival with irregular and very slow cleavages proceeded up to 80 hours.

Olsson (1965) noted that actinomycin C disturbed the "ordered mesenchyme" when administered at the blastula stage (256–512 cells) in *Echinus esculentus*.

One of the most striking evidences of differentiation following the onset of gastrulation is the formation of spicules. According to Okazaki (1965) the primary mesenchyme cells contribute to the elaboration of a spicular matrix within which a calcareous granule is elaborated. With actinomycin blocking at the blastula, the mesenchyme fails to form (Gross *et al.*, 1964) and thus the lack of a skeleton in the actinomycin D environment is explained. In *P. lividus* Lallier (1963a) observed that while primary mesenchyme cells did develop in blastulae (actinomycin = 10 μg./ml.), no progress beyond the morula and no spicules occurred.

If the embryos were washed, survival was enhanced, but migration of the primary mesenchyme into the blastocoele was delayed (Markman, 1964). Thus the actinomycin block to skeletal formation may lie in the inhibition of the elaboration of a spicular matrix by the primary mesenchyme cells. The defective arm formation is readily explained by Hörstadius' (1949) observation that the arm has to be predetermined in the ectoderm before the spicule reaches the ectoderm and the skeletal rod is needed to exact an influence to form an arm.

Although the level of actinomycin D (40 μg./ml.) used by Guidice and Hörstadius (1965) on *P. lividus* was higher than our levels (8 μg./ml.) the sequelae (blastular arrest, defective arm formation, and inhibition of skeletal formation) were similar.

Lallier (1963a, 1963b) and Markman and Runnström (1963) point out that actinomycin (like lithium) enhances the vegetalizing properties of the embryo. Runnström and Markman (1966) also cite that exposure to actinomycin D (12.5 μg./ml.) at the 8-cell stage for 5 hours causes animalization which is a "secondary effect of retarded gastrulation," and that lithium (0.033 *M*) to some extent counteracts this.

By now an enormous literature has grown on the biochemistry of the actinomycin D effect and no attempt will be made to survey the literature systematically. The bulk of work leads to the conclusion that the effect of actinomycin is due to the irreversible complexing with guanine-containing binding sites in DNA (Goldberg *et al.*, 1962; Kahan *et al.*, 1963). Thus the mechanism of inhibition of mRNA synthesis is brought about by the preferential binding of actinomycin to the primer DNA excluding a competition with precursors or a direct effect on RNA polymerase (Cavalieri and Nemchin, 1964). The biochemistry of actinomycin, and the complexing of it with DNA and purines, and the correlation of the structure and function of its complexes are beautifully demonstrated by Reich (1963, 1964).

Observations of the actinomycin D effect on echinoids on a molecular level abound since this compound has yielded a rich harvest of information on sequential biochemical events in development. To illustrate briefly, Gross and Cousineau (1963, 1964) noted in *Arbacia punctulata* that actinomycin treatment inhibited RNA turnover, but that the incorporation of amino acids into protein was unaltered and DNA synthesis continued although at a slower rate. In *Paracentrotus lividus,* Brachet (1963) and Brachet *et al.* (1963) observed that while actinomycin D slightly delayed cleavage, it did not inhibit the incorporation of C^{14}-leucine into proteins. Recently, Billiar *et al.* (1966) pointed out that actinomycin D inhibited protein synthesis in *A. punctulata* noticeably only after the early blastula stage.

In our studies the only agent in reasonably physiologic levels which appeared to ameliorate slightly the actinomycin D effect was DNA (0.2 mg./ml.). Thymine

(0.05 mg./ml.) merely enhanced survival while the effect of exogenous RNA was inconclusive. In experiments on *P. lividus,* Lallier (1963a, 1963b) demonstrated that 0.5 mg./ml. of DNA was protective against 10 μg./ml. of actinomycin D, but was ineffective if higher doses of actinomycin were used.

Kersten *et al.* (1960) indicated that purines and pyrimidines could displace actinomycin from the site in the living cell, and as a rule, the addition of DNA can ameliorate the actinomycin D effect, the actinomycin reacting directly with the DNA. According to Hurwitz *et al.* (1962) the inhibition of RNA polymerase by the antibiotic may be reversed by increasing the concentration of DNA. Yeast RNA at 100 times the concentration of DNA is only partly effective in relieving the inhibition (Goldberg and Rabinowitz, 1962). In ascites cells some of the inhibiting effects of actinomycin D (0.1 μg./ml.) are ameliorated by the addition of 200 μg./ml. DNA (Niu, 1963).

An interpretation of the enhancement of survival in actinomycin D by thymine cannot be provided at present. Setlow *et al.* (1963) demonstrated that one thymine dimer per 350 μ strand of DNA acted as a block to further DNA synthesis. In *E. coli* the block was permanent, while in other cells, it was only temporary. Kersten *et al.* (1960) indicate that thymine, as well as RNA could somewhat ameliorate the actinomycin block in *Neurospora crassa.*

Wheeler and Bennett (1962) suggested that actinomycin was preferentially fixed by the DNA of nucleoli. And in 1963 Reynolds *et al.* (1963a) reported a unique nucleolar effect of actinomycin D on Chang liver cells in culture, that of a separation of the pars amorpha and nucleolonema into "nucleolar caps."

Since the presence or absence of organized nucleoli in early echinoderm cleavage has not been fully explored, the pin-pointing of a cellular organelle as the target of actinomycin D, other than to a DNA-containing structure, is premature.

It is suggested that where these low concentrations of actinomycin D are eliciting a morphological response that there is a limited and differential binding of DNA by the antibiotic, and thus a differential synthesis of mRNA. Since skeletal formation and the formation of arms in the plutei are inhibited, it seems likely that the binding of DNA sites by actinomycin D is initially at very specific sites.

4-nitroquinoline N-oxide

The effects of 4-NQ on cell metabolism are many. According to Fukuoka *et al.* (1959) and Ono *et al.* (1959) in mouse ascites cells there was an inactivation of —SH enzymes, a subsequent inhibition of glycolysis, and as a result, a reduction in adenosinetriphosphatase, and thus no regeneration of ATP. Accordingly, the uptake and incorporation of amino acids into protein and the uptake of P^{32} into nucleic acids were depressed. Simply stated, the consequence of decreased DPN and ATP levels was an inhibition of protein and nucleic acid synthesis.

Endo *et al.* (1959) reported that 4-NQ produced intranuclear inclusion bodies in Chang liver and mouse kidney cells. Moreover, the cytological effects of 4-NQ (10^{-5} *M*) (Reynolds *et al.,* 1963a) in that in both instances there was a decrease in nucleolar size, a fusion of nucleoli, and separation of the pars amorpha and nucleolonema to form nucleolar caps (Reynolds and Montgomery, 1964; Reynolds *et al.,* 1964).

It was also proposed by Reynolds *et al.* (1964) that 4-nitroquinoline N-oxide might have the same specific effects on the DNA-dependent RNA production as actinomycin D. Our work suggests that the powerful mitotic arrest produced by 4-NQ on *Arbacia* embryos should be investigated by determining the incorporation of nucleic acid and protein precursors.

Since 4-NQ acted as a strong inhibitor of gastrulation (block at the blastula) at 5 μg./ml., its arresting qualities appear similar to those of actinomycin D. On the other hand, the viability of sperm at quite high concentrations of 4-NQ (0.125 mg./ml.) is of considerable interest. Obviously, the metabolic event which is blocked in the cleaving zygote is inoperable or most likely lacking in the sperm cell. Troll (personal communication) comments that methods which give evidence of RNA polymerase in *Arbacia* eggs do not reveal the presence of this enzyme in sperm cells. This negativity may, of course, be due to technical difficulties. Since there is a paucity of information about sperm nuclear enzymes, speculation about the lack of effect by 4-NQ is hampered. Reviews on general biochemical events (Monroy and Maggio, 1964), actinomycin D and RNA polymerase interaction (Reich and Goldberg, 1964), and the role of nucleic acids and sulfhydryls in echinoid morphogenesis (Brachet, 1964) are helpful only in elucidating these phenomena in eggs or cleaving zygotes.

Thus 4-nitroquinoline N-oxide may prove to be as effective a tool to probe the molecular aspects of sea urchin development as actinomycin D. Its ineffectiveness on sperm viability and inhibiting effect on zygote development are quite striking.

Coumarin

Coumarin is one of the naturally occurring active agents that is found in seeds and spores and takes its place among the native mitotic poisons such as the alkaloids and essential oils. According to Goodwin and Tanes (1950) coumarin should be thought of as an inhibitor rather than a toxic substance. The review by Soine (1964) lists some 31 different physiological activities of coumarins and indicates that many more may be forthcoming. In its natural state coumarin functions as a plant growth regulator, possibly at the level of the cytochromes.

Coumarin has long been recognized as an inhibitor of seed germination (Sigmund, 1914). Recent work attributes a growth-stimulating quality to coumarin (Knypl, 1964a, 1964b). Knypl (1964b) suggests that coumarin acts either through changes in the mechanical properties of the cell wall or through the metabolism of cells. He also indicated the possibility of a coordination of mRNA synthesis by coumarin (Knypl, 1965).

Since coumarin is a plant product its use on animal tissues has not been extensive aside from the studies on warfarin, a coumarin derivative. In rats, coumarin compounds generally antagonize vitamin K, uncouple oxidative phosphorylation, and thus affect the energy supply of the cell (Martius, 1961). By uncoupling oxidative phosphorylation, protein synthesis may be inhibited. One of the more recent accounts by Pool and Borchgrevink (1964) reports on warfarin as inhibiting the incorporation of amino acids into protein of the liver.

In the present experiments on sea urchin embryos, the mitotic inhibition by coumarin was demonstrated in the paucity of cells in the 40-hour-old embryos. In

addition, coumarin specifically and irreversibly interfered with the proper gastrulation and differentiation.

Cornman (1947) pointed out that coumarin suppressed the spindle, effected a splitting and shortening of chromosomes, and also prevented the inception of mitosis.

Because its full range of physiological activities may be so widespread (cytogenic, cytological, estrogenic, etc.; Soine, 1964), it is doubtful that its precise metabolic effect in *Arbacia* can be at present pinpointed.

Mescaline

The area of pharmacological effects of mescaline is a much more intensively studied one than that of the cellular role. Upon administration, oxidation of lactate, pyruvate, glutamate, and glucose is inhibited in guinea pig brain (Neff and Rossi, 1963). Whether injected intravenously (Neff *et al.*, 1964) or given orally (Charalampous *et al.*, 1964) to mammals it is agreed that it is deaminated to a physiologically inactive compound, 3, 4, 5-trimethyoxyphenylactic acid. It also has an inhibiting effect on the histaminolytic power of diamine oxidase (Carlini *et al.*, 1965).

Because of its striking neurological involvements in man, the cytological effects of mescaline have been noted principally in vertebrate tissues. A cytotoxic effect in tissue culture was reported by Painter *et al.* (1949) for a variety of substance that influence the activity of the nervous system. Lettré and his associates noted that 5 μg./ml. inhibited cell division and brought about vacuolization within the plasma membrane of chick fibroblasts (Lettré, 1948; Lettré and Albrecht, 1941, 1943; Lettré *et al.*, 1941). A direct cellular response as chromophobia of neurons, clumping of Nissl in neuroglia, etc., has been reported by Cazzullo (1963). Hoffer and Osmond (1960) state that it becomes bound to proteins of mitochondria and microsomes. It is also bound strongly by nuclei, weakly by microsomes and may be incorporated into tissue proteins (Neff and Rossi, 1963).

In our studies it was clear that in low concentrations of mescaline, development was slowed down. At the pluteus stage there is striking evidence of the inhibition of growth of the arms and the spicules, especially since a minuscule skeleton is formed in a normal-sized larva. The fact that the mescaline effect is not reversible after gastrulation suggests that a permanent binding or inhibition has occurred. The mechanism of action remains unclear but may be due to an inhibition of some step in the oxidative pathway. To illustrate, Zeuthen (1953) summarized the evidence to conclude that oxygen consumption in echinoids increased just about the time that primary mesenchyme cells appeared. Since the primary mesenchyme cells play a role in the formation of the spicular matrix (Okazaki, 1965) and since mescaline inhibits some oxidative step (Neff and Rossi, 1963), the miniaturization of the skeleton may be explained.

Thalidomide

One of the problems in administration of thalidomide is its insolubility and because of this, it is generally administered as a suspension rather than a true solution. Even without attempts at solubilization, at physiologic levels it decom-

poses spontaneously to give a mixture of compounds (Williams, 1963). Therefore, it was used in the present experiments in a solution that was often a suspension at concentrations about 0.02 mg./ml. to 0.1 mg./ml. Critical periods in *Arbacia* development during which the thalidomide effect could be evident presumably are: the initiation of cleavage, blastulation, gastrulation, and the inception of differentiation. It was found, however, that these stages were not subject to major disruptions, merely delay. The thalidomide effect, in fact, appeared to slow up at a later stage when the plutei began to show elongation of arms. Another specific effect was the persistence of the fertilization membrane in three-day-old plutei. This indicates that the hatching enzyme which is activated at the blastula (Kopac, 1941) is affected or the susceptibility of the fertilization membrane has been altered. According to Hallberg (1964) the hatching enzyme is present but inhibited in the unfertilized egg; therefore, thalidomide appears to prevent the release of this inhibition or may bring about a denaturation of the membrane which renders it undigestible by the hatching enzyme.

The mechanism of thalidomide action in disrupting normal echinoid embryogenesis is not fully elucidated. For protozoan inhibition, Frank *et al.* (1962, 1963) suggest that thalidomide interferes with the synthesis of diphosphopyridine nucleotides or the reduction of the cytochromes, or it may act at the point where adenine dinucleotide (NAD) is synthesized, or it may interfere with the utilization of NAD and vitamin K in cellular oxidations. Boylen *et al.* (1963) introduce evidence that thalidomide interferes with glutamine metabolism.

Miller (1963) puts the activity of thalidomide as an inhibitor of RNA and protein synthesis, while Vasilescu *et al.* (1964) report that thalidomide interferes with the metabolism of nucleic acids and alters their structure. In our experiments, RNA, RN-ase, DNA, and DN-ase enhanced rather than alleviated the retarding effects of thalidomide. Therefore, it is suggested that a strong binding of thalidomide or one of its degradation products to some cellular organelle occurred, or it induced an aberration in some biosynthetic pathway, and as a consequence normal embryogenesis was altered. The most plausible explanation is a block in the metabolism of glutamine.

Conclusions

For the early work on metabolic activities and cleavage of *Arbacia punctulata* referral is made to the review of Krahl (1950). The bibliography assembled by Harvey (1956) is highly relevant, while the more recent aspects of echinoid development are compiled by Monroy and Maggio (1964).

One other aspect of echinoid development needs to be considered in light of the dramatic exogastrulation elicited by cobalt. Runnström (1928) developed the concept that there are two potentialities in the sea urchin egg, one animal, the other vegetal, each occurring at the respective poles. During normal differentiation, these interact in a controlled manner. By altering the amounts of animal or vegetal blastomeres or by disrupting the metabolism of one of the potentialities a zygote can be directed into animalization (hyperdevelopment of ectodermal structures), or into vegetalization (hyperdevelopment of entomesodermal structures). The first striking example of vegetalization was that of Herbst (1892), which was induced by lithium ions. Various alterations in the direction of animalization or

vegetalization by ions, metabolites, etc., have been described, and reviews on this topic have been admirably put together by Ranzi (1962) and Lallier (1964a).

Ranzi (1962) advances a scheme for echinoid development based upon protein denaturation. Presumably, at the animal pole at fertilization, certain denatured proteins set into play the process for animalization. The proteolytic enzymes (ficin, trypsin, chymotrypsin) are animalizing (Hörstadius, 1949, 1953; Moore, 1952). Lallier's (1964a) view is that embryogenesis is directly affected by the inhibiting action of animalizing or vegetalizing agents or *via* "the formation of inhibitors of the synthesis of specific proteins."

In analyzing the effects of a substance on embryogenesis one should exclude substances that are ineffective at moderate concentrations and toxic with massive dose. These generally appear to elicit no graded dose-dependent response. No morphological changes occur, merely death and subsequent lysis of the blastomeres —as was found with sodium taurocholate. Toxic substance may be distinguished as those which show little or no effect below a certain level but exert an overall lethal effect at the proper (usually small) dose. They may act on any one, or on several cellular systems, bringing about complete cessation of function. The effect may be physicochemical as denaturation of proteins by alcohols. Enzymes, especially proteolytic ones, may fit into this category, especially if they penetrate.

Metabolites as normal constituents of the cell should be considered in their own category, that of being part of the cell economy. Herein would be included the enzymes, RN-ase and DN-ase and their respective substrates, RNA and DNA. Their effects would be analyzed in terms of the molecular biology of the cell.

Mitotic inhibitors, whether they act on the cytosome or on the nucleus, may bring about mitotic arrest or retard mitosis. A true mitotic retardant would give a dose-dependent inhibition; not so a mitotic arrestor. Thalidomide was a mitotic retarder. In the category of mitotic inhibitor would fall substances which slow down cell division and, in addition, alter the direction of morphogenesis. For example, cobalt may be considered genotropic in the sense that it disorients development and reorients it in another direction, *i.e.* toward exogastulation. A substance such as actinomycin D, however, which retards cleavage in low doses but firmly arrests it in higher levels and halts differentiation, could be genostatic, *i.e.,* the development of spicules and arms is totally inhibited. The compound, 4-nitroquinoline N-oxide, is genostatic; mescaline and coumarin most likely are genostatic as well.

The action of mitotic retarding agents would be reversible by changing the medium. The effect of genostatic and genotropic substances might be reversible, but irreversibility of action would be characteristic of toxic substances, and of genostatic and genotropic substances past the "point of no return."

Referral is made to Goodwin's (1963) analysis of control mechanisms. He states that there are three levels at which metabolites alter the activities of macromolecules by interaction: at the DNA level which would affect either DNA or RNA synthesis, at the messenger RNA level which would affect protein synthesis, and at the protein level which would affect the enzymes or the physical characteristics of proteins.

Dettlaff's (1964) review on embryonic development, analyzed from the point of view of mechanics of cell division, duration of periods of cleavage, periodicity of morphogenetic functions, etc., is also highly relevant.

Summary

1. For clarity, brevity, and accuracy in comparisons, a series of stages in the early development of the sea urchin, *Arbacia punctulata,* is presented. Each major morphological event is identified by a symbol.

2. Concentrations of ethanol and methanol of 1% or less have no visible effects on embryogenesis except for a slight growth retardation in ethanol. At 5% development was arrested at the morula stage, while at levels greater than 5% fixation of the cells occurred at the 2-cell stage.

3. The effects of papain (1 mg./ml.) on fertilized eggs were similar to but harsher than those of trypsin in arresting development and effecting dispersal of blastomeres. The fertilization membrane, however, was more resistant to dissolution than in trypsin. Upon lysis of the fertilization membrane, naked larvae often fell apart to blastomeres.

4. Sperm remained motile for several hours in pronase solutions (0.25 mg./ml.) whereas egg cells showed striking cortical changes followed by cytolysis. The fertilized egg, however, was more resistant and underwent cleavage in solutions 10 times more concentrated. Pronase often lysed the fertilization membrane within two hours post-fertilization; subsequently, separation of blastomeres occurred. When the membrane remained intact, normal plutei developed. It is suggested that pronase is a more suitable cell-dispersing enzyme than trypsin.

5. Sodium taurocholate was ineffective at 0.2 mg./ml. but at 2 mg./ml., by virtue of surface and internal changes (increasing lipolysis), brought about a rapid death to zygotes.

6. Exogenous DNA, rather than enhancing cleavage, causes a growth retardation at 0.1 mg./ml. with some lethal effects. Differentiation of mesodermal structures, however, continues although the pluteus shape is inhibited. The consensus is that nuclear aberrations are produced.

7. In general, the addition of DN-ase elicits a retardation of activity and cleavage in *Arbacia.* A concentration of 0.2 mg./ml. brings about death in 48 hours without any morphogenetic alterations. These effects may be attributed to a depletion of DNA by the enzyme.

8. At 0.1 mg./ml. and greater of RNA, an inhibition of cleavage is characteristic, but no specific malformations develop. Small levels of RNA (10 μg./ml.) accelerate slightly the rate of early cleavage, probably by a contribution to the cytoplasmic pool of RNA from which it is metabolized.

9. When physiological doses of RN-ase (0.1–0.12 mg./ml.) of RN-ase are added to sea water, *Arbacia* embryos exhibit only a slight retardation in rate of cleavage and a sluggishness in movement, and pass successfully through gastrulation. Larger amounts are cytotoxic, probably due to a massive degradation of cellular RNA.

10. Thymine, in concentrations of 0.04 mg./ml. to 0.4 mg./ml., inhibits development, the effects increasing with increasing doses. The block at gastrulation is striking at 0.4 mg./ml. Recovery by washing is rapid.

11. Cobalt chloride and cobalt glycine are lethal to *Arbacia* embryos at the blastula (0.2 mg./ml.) and at the morula (1 mg./ml.). In below-lethal levels (0.01 to 0.02 mg./ml.), the vegetalization-radialization is striking. Cleavage planes are disrupted, resulting in rough-surfaced embryos. The drastic aberration,

however, becomes evident at gastrulation when bloated embryos develop as exogastrulae. The skeleton is miniaturized. RNA and RN-ase very slightly ameliorate the effect, but RNA and DNA and DN-ase do not. Washing within five hours of immersion prevents exogastrulation.

12. The low solubility of actinomycin D in sea water and its slight precipitation upon standing must be considered in assaying for its dose-dependent retardation of cleavage and differentiation in sea-urchins.

13. Accordingly, 4 μg./ml. of actinomycin D effectively proves to inhibit full development beyond the blastula. The sequelae, blastular arrest, inhibition of spicule and arm formation, are well defined at 8 μg./ml. but this constitutes a toxic overdose. The first morphological evidence, aside from retardation of cleavage, was a disruption in the alignment of blastomeres, even before the morula stage. Reversal of the effect by washing, DN-ase, RNA or RN-ase was not obtained, but some amelioration was provided by DNA or thymine. Because of these events the binding of actinomycin probably occurs at specific sites.

14. Similar to actinomycin D, at 5 μg./ml. 4-nitroquinoline N-oxide, acted as a powerful inhibitor of gastrulation. Sperm cells, however, were viable for several hours at 0.0125 mg./ml. It is suggested that the metabolic block is inoperable in the sperm. The mechanism, inhibition of —SH groups or lack of RNA polymerase in sperm cells, needs to be investigated.

15. Coumarin (*ca.* 0.5 mg./ml.) can effectively act as a block to gastrulation. Although development is highly abnormal, the overall activity, swimming in eccentric orbits, is not curtailed. Which of the multiple physiological effects of coumarin is operable cannot be stated.

16. Mescaline at 1 mg./ml. is lethal to embryos at the gastrula stage. The most striking effect of mescaline (0.1 mg./ml.), aside from its growth-retarding one, is the production of a miniaturized skeleton in a normal-sized pluteus larva.

17. In non-lethal concentrations (0.02 to 1 mg./ml.) thalidomide permits normal although slower morphogenesis through the pluteus. The retardation, however, is not reversible by washing or the addition of RNA, RN-ase, DNA, DN-ase, etc. In many instances the fertilization membrane persisted about the larvae.

18. In evaluating the overall developmental effect of compounds on embryogenesis, substances may be identified, with some degree of overlapping, as: normal metabolites, animalizing, vegetalizing, or radializing agents, as mitotic arrestors or inhibitors, genotropic or genostatic or toxic.

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